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Efeitos agudos e crônicos de suplementação com diferentes doses de vitamina A sobre parâmetros de estresse oxidativo em pulmões de ratos

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Livros Grátis

Dedicatória

Dedico este trabalho a toda minha família, que sempre me inspirou e incentivou em todos os momentos a estudar e buscar, através do conhecimento, o reconhecimento.

Agradecimentos

RESUMO

A vitamina A e seus derivados, os retinóides, participam de processos celulares tais como desenvolvimento, proliferação, diferenciação e apoptose. Os efeitos da vitamina A, em sua maior parte, são atribuídos à ativação de receptores nucleares da família dos receptores esteróides, conhecidos como RAR e RXR. No entanto, o excesso de vitamina A ou de retinóides na dieta ou em uso terapêutico, pode ser teratogênico. Diversos trabalhos tem demonstrado *in vitro* e *in vivo* que a vitamina A e seus metabólitos são moléculas redox ativas, isto é, podem agir como pró-oxidantes ou antioxidantes, dependendo da concentração e do ambiente em que se encontram. Os objetivos deste trabalho foram investigar possíveis alterações no ambiente redox do pulmão de ratos Wistar tratados com vitamina A. Neste trabalho, foram utilizados ratos Wistar machos adultos – 90 dias (no início do tratamento), que foram tratados por 3, 7 ou 28 dias com vitamina A na forma de palmitato de retinol (Arovit®) nas doses de 1000, 2500, 4500 ou 9000 UI/kg/dia via intra-gástrica (gavagem). Verificamos um aumento significativo nos níveis de marcadores de estresse oxidativo (carbonilação de proteínas, peroxidação lipídica e diminuição no conteúdo de tióis reduzidos) além de modulação da atividade de enzimas antioxidantes em todos os períodos analisados, tanto nas doses consideradas terapêuticas que são as doses de 1000 e 2500 UI/kg/dia quanto nas doses excessivas 4500 e 9000 UI/kg/dia. Então, a partir destes resultados e de outros dados já reportados na literatura, acreditamos que seja necessária uma maior cautela no uso de vitamina A tanto a nível de suplementação quanto a nível terapêutico, visto que ela na forma que foi utilizada nesse trabalho pode ser adquirida livremente em farmácias sem a necessidade de receita médica.

ABSTRACT

Vitamin A and its derivatives, the retinoids, participate in cellular processes such as growth, cell division and apoptosis. The effects of vitamin A , in part, is ascribed to gene transcription mediated by nuclear receptors from the family of the steroid receptors, known as RAR and RXR. However, excessive vitamin A, or its retinoids, in the diet, or by therapeutic use, may be theratogenic. Diverse works have demonstrated *in vitro* and *in vivo* that vitamin A and its metabolites may be pro-oxidant or antioxidant. In this work, we aimed to investigate alterations in the redox environment of the lung of the rats Wistar treated with vitamin A. Here, we have utilized adult male Wistar rats (90/days old) that were treated for 3, 7 or 28 days with vitamin A as retinol palmitate at 1000, 2500, 4500 or 9000 IU/kg/day intra-gastrically (gavage).We have verified an increased level of oxidative stress markers (protein carbonylation, lipid peroxidation, and decreased protein and non-protein thiol content) and antioxidant enzymes activities modulation after any period of treatment, in all periods analyzed, both doses that are considered therapeutic doses of 1000 and 2500 IU / kg / day and in excessive doses 4500 and 9000 IU / kg / day. Then, regarding the results obtained in this work and from other reported data, we recommend caution in the use of vitamim A for supplementation or for therapeutic use.

LISTA DE ABREVIATURAS

ARAT- Acil-CoA:retinol aciltransferase

CAT- Catalase

CRALBP – Proteína celular ligadora de retinaldeído

CRABP – Proteína celular ligadora de ácido retinóico

CRBP – Proteína celular ligadora de retinol

CREB – Proteína ligadora de elemento responsivo à nucleotídeo cíclico

CYP26- Citocromos P450 da família 26

Cu$^+$/Zn$^+$-SOD – Superóxido dismutase cobre/zinco

DNA – Ácido desoxirribonucléico

ERK ½- Cinases reguladas por sinal extracelular 1 e 2

ERO- Espécies reativas do oxigênio

GSH- Glutationa redutase

GPx- Glutationa peroxidase

H$_2$O$_2$ – Peróxido de hidrogênio

JNK – Jun cinase

LRAT – Lecitina:retinol aciltransferase

NO$^{\cdot}$- Óxido nítrico

O$_2^{\cdot -}$ - Ânion superóxido

OH$^{\cdot}$- Radical hidroxila

ONOO$^{\cdot}$- Peroxinitrito

p38MAPK - Proteína cinase ativada por mitógenos p38

RAR – Receptor de ácido retinóico

RALDH – Retinal desidrogenase

RBP – Proteína ligadora de retinol

REH- Retinol éster desidrogenase

RXR – Receptor retinóide X

1. INTRODUÇÃO

1.1 Vitamina A e os retinóides

A vitamina A (retinol) e seus análogos, conhecidos coletivamente como retinóides, exercem um papel essencial em processos de crescimento e diferenciação (Bollag 1983; Gudas 1994). Por esta razão, os retinóides são reconhecidos reguladores de funções associadas à divisão celular e diferenciação, tais como reprodução, desenvolvimento embrionário e crescimento. Além disso, essas moléculas também estão envolvidas na manutenção de processos fisiológicos tais como visão e funções motoras.

O termo geral "retinóides" compreende o retinol, considerada a "molécula-raiz" da família da vitamina A, bem como o ácido retinóico, que é formado intracelularmente através do metabolismo oxidativo do retinol, além de outros metabólitos naturais, como o retinaldeído. Além disso, vários análogos sintéticos com similaridade estrutural ou funcional ao retinol também são reconhecidos como pertencentes à grande família dos retinóides. Os retinóides naturais são compostos isoprenóides de 20 carbonos com um anel beta-ionilideno, uma cadeia lateral de carbonos contendo ligações duplas que possibilitam variadas configurações isoméricas, e um grupo funcional terminal em um dos três estados de oxidação(Bollag 1983).

A síntese *de novo* de retinol é restrita às plantas e a alguns microorganismos (Mercer, Davies *et al.* 1963). Animais obtêm vitamina A da Exemplos de pró-vitamina A são alguns compostos carotenóides, que são encontrados em diversos vegetais. O beta-caroteno, por exemplo, é convertido em

retinol através de dois passos enzimáticos na mucosa intestinal. Por outro lado, a principal forma de vitamina A pré-formada encontrada na dieta é o retinol esterificado a ácido graxos de cadeia longa (chamados genericamente de ésteres de retinol), obtido através de alimentos de origem animal (principalmente fígado, leite e derivados)(Olson 1996), além de alimentos processados industrialmente e em suplementos vitamínicos. Ésteres de retinol são hidrolisados no lúmen intestinal por enzimas pancreáticas e da mucosa intestinal. Após esses processamentos enzimáticos da vitamina A pré-formada e da pró-vitamina A no lúmen intestinal, o retinol livre é absorvido pelas células da mucosa e re-esterificado a ácidos graxos, geralmente saturados, de cadeia longa, no citoplasma das mesmas (através da ação da enzima lecitina: retinol aciltransferase, LRAT) (MacDonald and Ong 1988). Os ésteres de retinol resultantes desse processo são incorporados, com outros ésteres de lipídios neutros (por exemplo, triacilglicerídeos e ésteres de colesterol), nos quilomícronsm, e absorvidos através do sistema linfático(Harrison and Hussain 2001).

Durante o processamento das quilomícra pelos tecidos extra-hepáticos, algumas células absorvem ésteres de retinol liberados pela ação da lípase lipoprotéica (Miano and Berk 2000). As quilomícra remanescentes são, em seguida, captados pelo fígado, que é o principal órgão armazenador de vitamina A sob condições normais (Yost, Harrison *et al.* 1988). Neste órgão, o retinol é captado primeiramente pelos hepatócitos, onde uma hidrolase se ésteres de retinol (REH) hidrolisa esses compostos, gerando retinol livre. Este é complexado a proteínas citoplasmáticas ligadoras de retinol (*cellular retinol binding proteins,* ou CRBPs) (Thompson and Gal 2003).

Após esse processo de captação de retinol pelo hepatócito, o retinol em excesso é transportado por difusão para as células hepáticas estreladas, onde é re-esterificado pela ação da acil-CoA:retinol aciltransferase (ARAT) e pela LRAT, sendo armazenado em gotas lipídicas citoplasmáticas, juntamente com outros lipídios neutros. Em condições normais, 80% do retinol hepático em um indivíduo normal é encontrado nas células estreladas, sendo o restante encontrado nos hepatócitos. O retinol hepático é mobilizado através da ação de uma REH nas células estreladas hepáticas, que hidrolisam os ésteres de retinol e os liberam para serem complexados a proteínas ligadoras de retinol plasmáticas (*retinol binding proteins*, RBPs), nos hepatócitos, para secreção na circulação(Soprano, Gyda *et al.* 1994).

O transporte de retinol para os tecidos extra-hepáticos, como por exemplo os pulmões, é realizado principalmente pelas RBPs. Dois modelos de captação celular de retinol foram propostos; o modelo da difusão propõe que o passo-limitante na captação de retinol é a lenta dissociação do *holo*-complexo retinol-RBP no ambiente extracelular (Hussain, Kedees *et al.* 2001), enquanto que o modelo da captação mediada por receptor sugere que este é um processo específico, mediado pela interação da RBP com um receptor de membrana (Ross 1993). Uma vez dentro da célula, o retinol complexa-se novamente com proteínas ligadoras de retinol (CRBPs)

O retinol citoplasmático tem diversos destinos metabólicos, que variam principalmente de acordo com a função celular. Nos hepatócitos, diversos tipos de metabólitos diretos do retinol são formados através da ação de enzimas do complexo citocromo P450 (Noy 2000). Alternativamente, o retinol pode ser oxidado a 11-*cis*-retinal, composto de importância central no ciclo visual, pela

retinol-desidrogenase(RDH). O 11-*cis*-retinal pode permanecer no citoplasma complexado a proteínas de função homóloga às CRBPs (chamadas *cellular retinaldeyde binding proteins,* CRALBPs) ou ser oxidado a ácido retinóico pela retinal desidrogenase (RALDH). O ácido retinóico citoplasmático também se encontra associado a proteínas ligadoras específicas (*cellular retinoic acid binding proteins,* CRABPs), sendo que formas plasmáticas dessa proteína também já foram identificadas (Ross 1993). A degradação enzimática do ácido retinóico é catalisada por enzimas da família 26 do grupo das citocromos P450 (denominadas CYP26). Além disso, o ácido retinóico, que é normalmente encontrado na forma "todo-*trans*", pode ser isomerizado em formas *cis*, como o ácido retinóico 9-*cis*, e o significado fisiológico exato desta transformação ainda é bastante debatido.

1.2 MECANISMOS DE AÇÃO

Em 1987, descobriu-se que os retinóides exercem suas funções através da sua ligação a receptores nucleares específicos (RXR e RAR) que, por sua vez, agem como fatores de transcrição, regulando, portanto, a expressão gênica de seqüências de DNA-alvo àquelas moléculas sinalizadoras. Por esta razão, estes compostos têm sido definidos, por muitos autores, como hormônios.

Além da forma tradicionalmente aceita de sinalização mediada por vitamina A, tem se demonstrado que a vitamina A pode atuar, em nível celular, de maneira independente do núcleo, por meio de uma ação não-genômica. Há um consenso na literatura de que a ação não-genômica da vitamina A, por definição, não envolveria transcrição gênica mediada por receptores retinóides (RAR – RXR). No entanto, isso não significa que esses receptores não estejam envolvidos

nesse fenômeno, como sugerem alguns autores. Inclusive, teoricamente, seria até possível que um dos efeitos, em longo prazo, da ativação de rotas de sinalização não-genômica da vitamina A, fosse a modulação da própria modulação da transcrição gênica mediada por esses receptores. Foi demonstrado, por exemplo, que certas rotas de sinalização podem alterar diretamente a atividade desses receptores, como é o caso das rotas da p38MAPK, cdk e JNK (Bastien and Rochette-Egly 2004). Nosso grupo também demonstrou que a via de sinalização celular ERK ½ -CREB pode ser ativada por retinol em concentrações pouco acima do fisiológico no modelo experimental utilizado (Gelain, Cammarota *et al.* 2006). Portanto, essas ações não-genômicas da vitamina A parecem independer de sua ligação a receptores nucleares, mas o exato mecanismo de ação ainda merece ser invetigado.

Além disso, alguns dados da literatura incluindo trabalhos do nosso grupo, mostram que a vitamina A e alguns de seus derivados são moléculas redox ativas, ou seja, dependendo de algumas condições, podem reduzir ou oxidar outras biomoléculas. Dentre os efeitos observados, temos aumento na produção mitocondrial de radical superóxido, aumento nos níveis de marcadores de peroxidação lipídica e de carbonilação de proteínas, e modulação na atividade de enzimas antioxidantes, tais como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) (Gelain, Cammarota *et al.* 2006; de Oliveira, *et al.* 2008). Além desses dados envolvendo o balanço redox, também foi observado que a suplementação com vitamina A pode levar alterações comportamentais (de Oliveira, *et al.* 2008). Estes dados mostram que, em diferentes modelos experimentais, o tratamento com retinol/palmitato de retinol pode induzir pulsos

de estresse oxidativo, sendo que um pulso transiente na formação de espécies oxidantes é uma conseqüência deste tratamento *in vitro*.

1.3 RADICAIS LIVRES E ESTRESSE OXIDATIVO

Um radical livre é uma espécie química com um ou mais elétrons desemparelhados. As espécies químicas podem ser átomos, como hidrogênio ou cloreto, metais de transição, ou uma molécula onde o elétron desemparelhado esteja localizado no orbital externo. Este elétron desemparelhado confere uma reatividade relativamente alta a esta molécula, devido a uma grande tendência de esta adquirir ou perder um segundo elétron para este orbital (Halliwell 2006).

Radicais livres são escritos quimicamente com uma notação para a espécie química seguida de um ponto, o qual indica o elétron desemparelhado, por exemplo, o radical livre ânion superóxido: $O_2^{\bullet -}$

Quimicamente, radicais livres são caracterizados por sustentarem reações em cadeia, que se autopropagam, onde ou uma molécula reduzida perde ou uma oxidada ganha seu elétron para o radical livre, e tornando-se, agora, um radical livre, reagindo com outro composto químico, e assim por diante.

Classicamente, as reações de radicais livres são divididas em: a) reações de iniciação; b) reações de propagação; e c) reações de terminação. Nas reações de iniciação, um radical livre é formado a partir de espécies químicas não-radicais (e, portanto estáveis): $AB + C \rightarrow A^{\bullet} + D + E$. Nas reações de propagação, um radical livre, também chamado centro de reação, reage com uma molécula estável,

resultando em outro radical livre, ou centro de reação: $A^{\bullet} + CD \rightarrow AC + D^{\bullet}$. Nas reações de terminação, dois radicais livres cancelam seus elétrons desemparelhados formando um produto estável.

A reatividade química dos radicais livres é determinada pela molécula que carrega este elétron desemparelhado; conseqüentemente, a reatividade varia muito entre um radical e outro. Um modo de expressar e comparar a reatividade química destas moléculas é especificar a meia-vida das mesmas. Uma meia-vida curta indica alta reatividade, e o radical hidroxila ($OH^{\bullet}$) é o mais reativo dos radicais livres (sendo, então, o mais instável, ou seja, é aquele que reage mais rapidamente assim que formado).

Um dos principais radicais livres conhecidos é o ânion superóxido ($O_2^{\bullet-}$), que é produzido quando uma molécula de oxigênio é reduzida parcialmente, ou seja, quando recebe apenas um elétron, ao invés de receber dois elétrons. Caso haja redução completa como ocorre no complexo IV da cadeia respiratória, água é formada. Quantidades excessivas de $O_2^{\bullet-}$ levam a dano tecidual por induzir a produção de hidroxila derivado da reação com peróxido de hidrogênio (H_2O_2) com metais de transição Fe^{+2} ou Cu^{+2} – reação de Fenton. Além disso, ao reagir com óxido nítrico ($NO^{\bullet}$), $O_2^{\bullet-}$ forma peroxinitrito ($ONOO^{\bullet}$) que, por sua vez, pode gerar o radical nitrosila ($ONOOH$) que, ao se decompor, também forma o radical $OH^{\bullet}$.

Os radicais livres podem causar dano oxidativo aos componentes celulares como lipídios, carboidratos, proteínas e DNA devido a sua alta reatividade e natureza oxidante. No entanto, as células contam com defesas contra tais efeitos danosos daquelas moléculas. São as defesas antioxidantes, que podem ser tanto enzimáticas quanto não-enzimáticas. Entre as defesas enzimáticas estão as enzimas superóxido dismutase, catalase e glutationa peroxidase. O tripeptídio glutationa (na forma reduzida – GSH) e as vitaminas (originadas da dieta, como o ácido ascórbico, e a vitamina E, por exemplo) representam defesas antioxidantes não-enzimáticas.

As enzimas acima citadas são tidas como defesas antioxidantes primárias, ou seja, agem diretamente sobre a molécula do radical livre, antes que este possa vir a oxidar uma biomolécula. A enzima superóxido dismutase (SOD) apresenta quatro classes: Mn-SOD (localizada na matriz mitocondrial), Cu, Zn-SOD(citosólica), Ni-SOD e SOD extracelular. Todas estas formas de SOD agem sobre o radical $O_2^{\bullet-}$, transformando-o em peróxido de hidrogênio (H_2O_2) e oxigênio através da seguinte reação:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

Já a enzima catalase (CAT) age sobre o peróxido de hidrogênio gerado pela reação acima ou por outras reações, transformando-o em água por meio da reação:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

A enzima glutationa peroxidase (GPx) também atua sobre o H_2O_2, no entanto, por meio de um mecanismo diferente. A GPx participa de um ciclo redox junto da enzima glutationa redutase, onde GSH é usada, pela GPx, para transformar H_2O_2 em água; e NADPH é utilizado pela glutationa redutase para reduzir a glutationa oxidada, na primeira reação, em GSH novamente (Boveris 1998).

Estresse oxidativo é o termo utilizado em uma situação onde a formação de radicais livres excede a capacidade de transformação destas moléculas em outras não-oxidantes por meio das defesas antioxidantes. Neste caso, podemos dizer que ocorreu um desequilíbrio entre a formação de radicais livres e a atuação da defesa antioxidante. E isto pode ocorrer por diversos motivos, inclusive, inativação de enzimas como SOD e CAT por meio de reações destas com as próprias moléculas oxidantes, onde as enzimas perdem sua característica nativa e, conseqüentemente, sua função. Um exemplo é a inativação de catalase por $O_2^{\bullet-}$ (Shimizu, Kobayashi *et al.* 1984).

Dentro da célula, podem-se encontrar fontes de formação de radicais livres, tais como a cadeia transportadora de elétrons mitocondrial, onde a redução parcial do oxigênio dará origem ao $O_2^{\bullet-}$. Existem dois locais da cadeia transportadora de elétrons de onde os elétrons podem vazar, formando o $O_2^{\bullet-}$. O primeiro é a partir do complexo da coenzima Q e o outro é a partir do Complexo III.

Embora a mitocôndria seja o local mais importante de produção de radicais livres endógenos, existem outros locais onde estas moléculas oxidantes podem ser formadas. No citosol, por exemplo, a cascata do ácido araquidônico, que produz prostaglandinas e leucotrienos, pode formar espécies reativas do oxigênio (ERO) quando o lipídio metabolizado é liberado. Ainda, algumas isoenzimas do citocromo P-450 também estão descritas como produtoras de ERO (Halliwell 2006).

1.4 VITAMINA A E O TECIDO PULMONAR

A vitamina A influencia diretamente a maturação e diferenciação das células pulmonares. As células alveolares do tipo II são as células que secretam surfactante. Surfactante é uma mistura de fosfolipídeos com proteínas, o qual tem como função diminuir a tensão superficial das células pulmonares. A expressão de surfactante pelas células alveolares do tipo II é regulada de maneira dose-dependente por ácido retinóico. Além disso, a vitamina A é conhecida por preservar e manter a integridade do epitélio pulmonar. Durante episódios de injúria, a vitamina A é responsável pela proliferação das células alveolares do tipo II e de sua diferenciação em células alveolares do tipo I(Takahashi, Miura et al. 1993).

Já é relatado que a deficiência em vitamina A leva a uma diminuição no conteúdo de elastina nos pulmões, diminui a síntese de surfactante pelas células alveolares do tipo II, formação de áreas com líquido intersticial, efeitos esses que estão ligados diretamente a processos patológicos de doenças pulmonares. Entretanto, pouco se sabe sobre os possíveis efeitos da suplementação com

vitamina A (palmitato de retinol) em relação ao tecido pulmonar no que diz respeito ao ambiente redox deste.

2. OBJETIVOS DO TRABALHO

Objetivando analisar os efeitos de uma suplementação com vitamina A sobre o ambiente redox *in vivo*, decidimos realizar tratamentos de curta e de moderado-longa exposição com vitamina A (na forma de palmitato de retinol - Arovit®) em doses consideradas pela literatura como terapêuticas ou excessivas em pulmões de ratos Wistar machos adultos.

Como objetivos específicos, então, analisamos os efeitos da suplementação diária com vitamina A via intragástrica nas doses de 1000, 2500, 4500 ou 9000 UI/kg/dia por 3, 7 ou 28 dias sobre:

- Peroxidação lipídica, carbonilação de proteínas, estado redox de grupamentos sulfridril protéicos e não-protéicos no tecido pulmonar (capítulo I e II);

- Modulação das defesas antioxidantes enzimáticas: superóxido dismutase(SOD) e catalase (CAT) (capítulo I e II);

- Modulação no imunoconteúdo das enzimas antioxidantes: superóxido dismutase(SOD) e catalase (CAT) (capítulo I).

PARTE II

2. MATERIAL, MÉTODOS E RESULTADOS

Nesta parte do trabalho, apresentamos os resultados em forma de artigos científicos. Em ambos os artigos , o modelo experimental foi o mesmo: suplementação diária com vitamina A hidrossolúvel (palmitato de retinol - Arovit®) em doses terapêuticas (1000 e 2500 UI/kg/dia) e supra-terapêuticas (4500 ou 9000 UI/kg/dia) por dois períodos curtos (3 e 7 dias) e um longo (28 dias). O tratamento foi sempre realizado à noite, pois a vitamina A tem sua absorção aumentada quando administrada junto das refeições, e como os ratos são animais de hábitos noturnos, se alimentam principalmente à noite. Após 24 horas do último dia de tratamento os animais foram sacrificados e os pulmões foram retirados cirurgicamente e lavados exaustivamente em tampão fosfato,com pH 7,4 e gelado. Os pulmões, então, foram homogeneizados em tampão fosfato, com pH 7,4, e mantidos a -80°C até o dia das análises, que nunca pode exceder uma semana. Investigamos parâmetros de estresse oxidativo, como marcadores de dano oxidativo em biomoléculas (peroxidação lipídica, carbonilação de proteínas e estado redox de grupamentos tióis), atividade de enzimas antioxidantes (SOD, CAT) e imunoconteúdo das enzimas (SOD, CAT).

Capítulo I

"VITAMIN A SUPPLEMENTATION INDUCES OXIDATIVE STRESS AND DECREASES THE IMMUNOCONTENT OF CATALASE AND SUPEROXIDE DISMUTASE IN RAT LUNGS"

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VITAMIN A SUPPLEMENTATION INDUCES OXIDATIVE STRESS AND DECREASES THE IMMUNOCONTENT OF CATALASE AND SUPEROXIDE DISMUTASE IN RAT LUNGS

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□ *Lungs require an adequate supply of vitamin A for normal embryonic development, postnatal maturation, and maintenance and repair during adult life. However, recent intervention studies revealed that supplementation with retinoids resulted in a higher incidence of lung cancer, although the mechanisms underlying this effect are still unknown. Here, the authors studied the effect of vitamin A supplementation on oxidative stress parameters in lungs of Wistar rats. Vitamin A supplementation either at therapeutic (1000 and 2500 IU/kg) or excessive (4500 and 9000 IU/kg) doses for 28 days induced lipid peroxidation, protein carbonylation, and oxidation of protein thiol groups, as well as change in catalase (EC 1.11.1.6; CAT) and superoxide dismutase (EC 1.15.1.1, SOD) activities and immunocontents. These results altogether suggest that vitamin A supplementation causes significant changes in redox balance the free radical status in lungs, which are frequently associated to severe lung dysfunction.*

Keywords lung, oxidative stress, vitamin A

In the past few years, vitamin A (retinol) and its metabolites (retinoids) have been frequently suggested to be an important antioxidant for tissues such as lungs, liver, and heart [1]. In lungs, retinoids are essential for normal morphogenesis during the fetal period, for maturation and remodeling in the perinatal and postnatal periods, and for maintenance of the fully matured lungs [2]. Several studies suggested that β-carotene and vitamin A could exert cancer-preventive effects by a mechanism of free radical scavenging and/or detoxification [3]. In many developing countries, it is more common for infants, children, and adults to be provided with a single high-dose vitamin A (300 to 10000 IU/kg) supplement at regular intervals, with various

coverage rates [3a]. Adults ingested 5000 IU/day in average of vitamin A through diet. The physiological range for retinol in cells varies between 0.2 and 5 μM [3b].

However, the Beta-Carotene and Retinol Efficacy Trial (CARET), which halted a daily intervention with β-carotene and vitamin A, observed an increased incidence of lung cancer and mortality rate caused by this condition [4]. Besides, daily intervention with β-carotene (30 mg) combined with retinyl palmitate (25000 IU/kg) increased incidence of lung cancer and colorectal cancer in asbestos-exposed workers [5–7]

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the redox cell balance. When the balance between cellular antioxidants and ROS/RNS productions shifts toward oxidative stress, oxidants cause structural changes and/or degradation of nucleic acids, proteins, and lipids. ROS/RNS generation processes are mainly localized in cellular membranes. Therefore, it may be assumed that lipophilic antioxidants are the primary protectors against ROS/RNS [8]. It has been demonstrated that oxidative stress may contribute to the pathogenesis of diffuse lung diseases (DLDs) [9]. DLDs are a heterogeneous group of diseases with different etiopathogenesis, clinical course, prognosis, and degrees of pulmonary fibrosis.

Vitamin A is either antioxidant [10] or prooxidant, depending on its concentration. Previously, we showed that vitamin A induces protein carbonylation and lipid peroxidation, and that antioxidant enzyme activities are modulated by retinol [11–18] and all-*trans* retinoic acid [18, 19] treatments in cultured Sertoli cells. Rat liver mitochondria are vulnerable to retinol [20] and retinoids [21], which may induce the release of proapoptotic factors from mitochondria. Furthermore, supplementation with vitamin A also increases oxidative damage in cerebral structures and altered rat behavior [22–24].

Considering that retinol supplementation causes oxidative stress in cells and increases lung cancer incidence in humans, we decided to investigate the effects of vitamin A supplementation either at therapeutic (1000 and 2500 IU/kg) or excessive (4500 and 9000 IU/kg) doses upon parameters of oxidative stress rat lungs. We observed that vitamin A supplementation increases parameters of oxidative stress in lung.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (90 days old; 280 to 320 g) were obtained from our own breeding colony. They were caged in groups of 5 with free access to food and water and were maintained on a 12-h light-dark cycle (7:00 to 19:00 hours), at a temperature-controlled colony room ($23°C \pm 1°C$). These

conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH publication number 80-23, revised 1996). Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

Drugs and Reagents

Arovit (retinol palmitate, a commercial water-soluble form of vitamin A) was purchased from Roche. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A administration solutions were prepared daily, protected from light exposure and temperature.

Treatment

The animals were treated once a day for a period of 28 days. All treatments were carried out at night (i.e., when the animals are more active and eat a greater amount of food) in order to ensure maximum vitamin A absorption, because this vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0.15 M saline), 1000, 2500, 4500, or 9000 IU/kg of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.8 mL during each period of interest. Adequate measures were taken to minimize pain or discomfort.

Lung Extraction and Samples Preparation

The animals were sacrificed by decapitation at 24 hours after the last vitamin A administration. The lung was dissected out in ice immediately after the rat was sacrificed and stored at $-80°C$ for posterior biochemical analyses. The lungs were homogenized in 50 mM phosphate buffer (pH 7.0) and centrifuged ($700 \times g$, 5 minutes) to remove cellular debris. Supernatants were used to all biochemical assays described. All the results were normalized by the protein content using bovine albumin as standard [25].

Thiobarbituric Acid–Reactive Species (TBARS)

As an index of lipid peroxidation, we measured the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described [26]. Briefly, the samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 minutes. TBARS were determined spectrophotometrically by the absorbance at 532 nm. Results are expressed as TBARS/mg protein.

Measurement of Protein Carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) as previously described [27]. Briefly, proteins were precipitated by the addition of 20% TCA, redissolved in DNPH and the absorbance at 370 nm measured on a spectrophotometer. Results are expressed as nmol carbonyl/mg protein.

Measurement of Protein Thiol Content

Proteins thiol content in samples was analyzed to estimate oxidative alterations in proteins. Briefly, an aliquot was diluted in sodium dodecyl sulfate (SDS) 0.1% and 0.01 M 5,50-dithionitro-bis-2-nitrobenzoic acid (DTNB) in ethanol was added; the intense yellow color developed was measured on a spectrophotometer at 412 nm after 20 minutes. [28]. Results are expressed as mmol SH/mg protein.

Antioxidant Enzyme Activities Estimations

Catalase (EC 1.11.1.6; CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm on a spectrophotometer [29], and the results are expressed as units of CAT/mg protein. Superoxide dismutase (EC 1.15.1.1, SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation on a spectrophotometer using absorbance at 480 nm, as previously described [30], and the results are expressed as units SOD/mg protein. A ratio between SOD and CAT activities (SOD/CAT) was applied to better understand the effect of vitamin A supplementation on these 2 oxidant-detoxifying enzymes, which work in sequence converting superoxide anion to water [31]. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

Immunoblot

To perform immunoblot experiments, samples were lysed in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% [*w/v*] SDS, 10% [*v/v*] glycerol) and equal amounts of cell protein (30 μg/well) were fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. Protein loading and electroblotting efficiency were verified through Ponceau S staining, and the membrane was blocked in Tween–Tris-buffered saline (TTBS: 100 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin. Membranes were incubated overnight at 4°C with rabbit polyclonal antibody against CAT (dilution range 1:400) in the presence of 5% milk, SOD2

(dilution range 1:400), or anti-β-actin 1:2000, and then washed with TTBS. Anti-rabbit immunoglobulin G (IgG) peroxidase-linked secondary antibody was incubated with the membranes for additional 1 hour (1:5000 dilution range), washed again, and the immunoreactivity was detected by enhanced chemiluminescence using ECL Plus kit. Densitometric analysis of the films was performed with ImageQuant software. Blots were developed to be linear in the range used for densitometry. All results were expressed as a relative ratio between CAT and SOD2 immunocontent and the β-actin internal control immunocontent.

Statistical Analysis

Biochemical results are expressed as means $\pm$ standard error of the mean (SEM); P values of $<.05$ were considered significant. Differences in experimental groups were determined by 1-way analysis of variance (ANOVA) followed by the post hoc Tukey's test whenever necessary.

RESULTS

We observed an increased lipid peroxidation levels in the lung of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg ($P < .05$) (Figure 1A). This increase in lipid peroxidation levels in the lung of the rats that received vitamin A supplementation is dose dependent. Besides, protein carbonylation levels increased ($P < .05$) in the lung of the rats treated with vitamin A at all doses tested (Figure 1B); vitamin A supplementation at 1000 IU/kg increased lung carbonyl levels by 2-fold, and higher doses presented a similar effect. All doses were also observed to induce a significant decrease in lung protein thiol content in relation to control group, confirming that vitamin A supplementation exerted a pro-oxidant effect on lung proteins. Interestingly, the doses of 1000 and 2500 IU/kg presented the most pronounced effect on lung thiol levels, whereas the dose of 4500 IU/kg had a milder effect (Figure 1C). At 9000 IU/kg, the effect was not statistically different from the decrease in thiol content observed at 1000 and 2500 IU/kg.

We next investigated the activation status and immunocontent of the antioxidant enzymes SOD and CAT in the lungs of rats that received vitamin A supplementation. SOD activity increased ($P < .05$) in the lung of the rats that received vitamin A supplementation only at 4500 or 9000 IU/kg (Figure 2A). To investigate the mechanism of SOD activation, we performed the immunodetection of SOD protein in the lungs of the supplemented rats. Interestingly, SOD immunocontent decreased at 4500 or 9000 IU/kg (Figure 3A). This result indicated that the increase in SOD activity caused by vitamin A supplementation was not related to SOD protein synthesis. On the other hand, vitamin A supplementation induced a significant decrease ($P <$

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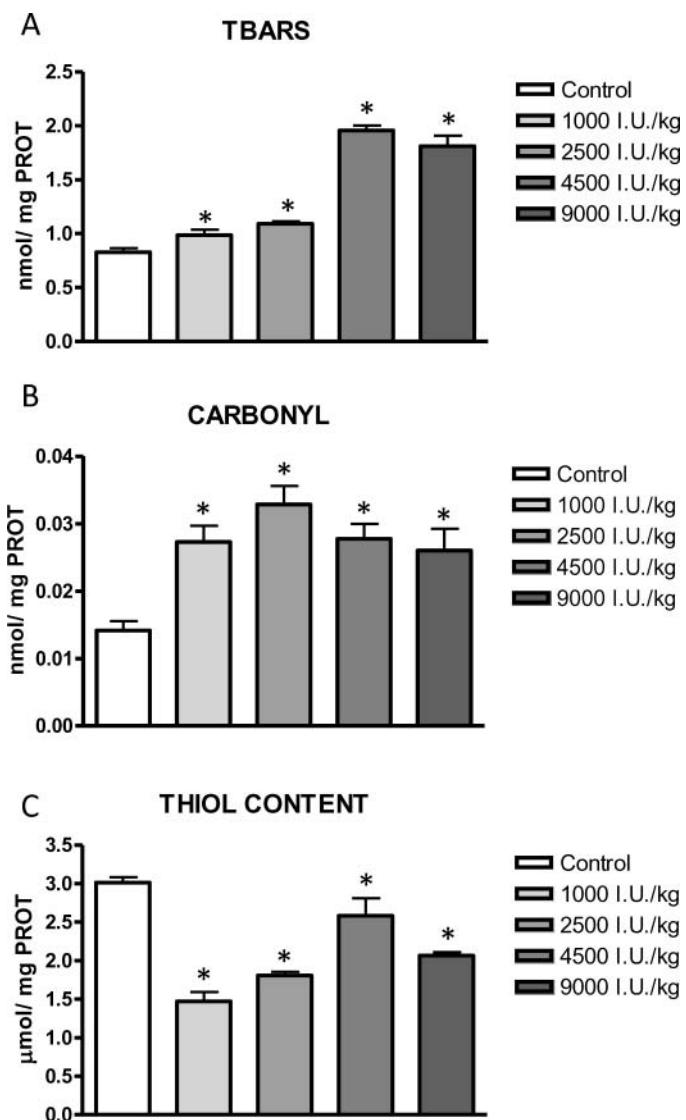
FIGURE 1 Effects of vitamin A supplementation on lipid peroxidation (*A*), protein carbonylation (*B*), protein thiol content (*C*) in the rat lung. Data are mean ± SEM (*n* = 7–12 per group). *P < .05 (1-way ANOVA followed by the post hoc Tukey's test).

.05) in lung CAT activity (Figure 2*B*). This effect was observed at all doses tested and the decrease was similar in all groups treated with vitamin A. Although all doses of vitamin A decreased CAT activity at the same extent, the lung immunocontent of CAT was observed to be decreased only at 4500 or 9000 IU/kg (Figure 3*B*). This decrease in CAT immunocontent by vitamin A supplementation was dose dependent. Besides, as depicted in Figure 2*C*, vitamin A supplementation at any dose induced an imbalance (P < .05) in lung SOD/CAT ratio. Altogether, these data indicate that vitamin A
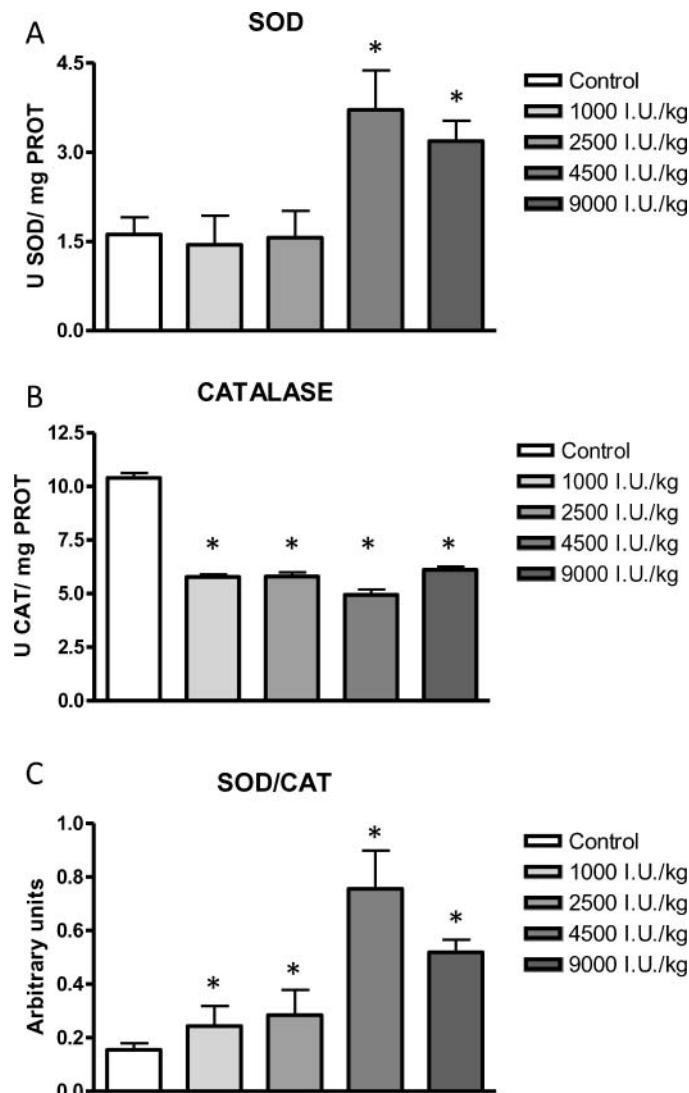
FIGURE 2 Effects of vitamin A supplementation on SOD (*A*), CAT (*B*), activity in the rat lungs. (*C*) The lung SOD/CAT ratio. Data are mean $\pm$ SEM ($n = 7$–12 per group). $^*P < .05$ (1-way ANOVA followed by the post hoc Tukey's test).

induce differential effects on the regulation of activity and protein synthesis/turnover of CAT and SOD, which probably contributes to the pro-oxidant effects reported in this work.

DISCUSSION

Vitamin A supplementation is commonly recommended for both nutritional and therapeutic purposes. The amount of vitamin A intake in such

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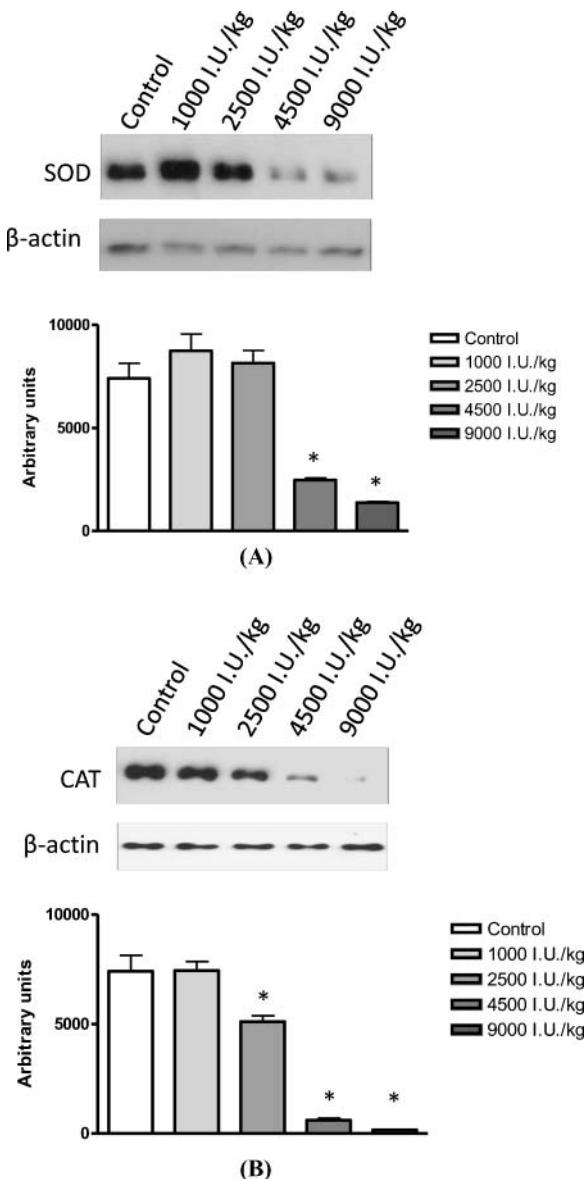
FIGURE 3 Effects of vitamin A supplementation on immunocontent of SOD (**A**) and CAT (**B**). Representative immunoblot and quantification values are shown. Data are mean $\pm$ SEM and were normalized against β-actin immunocontent ($n = 7$–12 per group). $^*P < .05$ (1-way ANOVA followed by the post hoc Tukey's test).

situations, however, varies according to multiple factors. It is commonly reported that daily nutritional supplements provide 5000 IU of vitamin A per day to adults, and 2500 IU for children, in industrialized countries, not considering the amount of vitamin A obtained from regular diet, which is already considered high in industrialized countries [3b]. In developing

28

countries where starvation is a public health concern, high doses of vitamin A are provided to children and pregnant women along with vaccines, and quantities such as 25000 to 200000 IU are provided within regular intervals [3b], which easily reach the range utilized in the present work (1000 to 9000 IU/kg). Thus, the doses studied here might be considered therapeutic, supplemental or excessive, depending on the administration protocol and the aim of the administration.

We observed that vitamin A treatment at different doses induced a prooxidant state in lung of adult rats. Vitamin A treatment not only increased lipid peroxidation and protein carbonylation, but also decreased protein thiol content. Moreover, the activities and immunocontents of CAT and SOD were also modulated by vitamin A supplementation.

We observed that vitamin A supplementation decreases CAT activity. It is known that vitamin A treatment increases superoxide anion (O_2^-) production in cell cultures and in vitro [20, 32]. O_2^- is a well-known CAT inhibitor [33, 34] and we observed here that CAT immunocontent levels decreased after vitamin A supplementation. Decreased CAT activity caused by enhanced H_2O_2 production is a possible mechanism. CAT changes enzyme conformation to the so-called compound II state during exposure to its own substrate H_2O_2 [34a]. In this conformation, CAT decreases its activity. Thus, during exposure to H_2O_2 that is generated at constant rates, CAT can reach a steady state in which most of it is inactive.

In addition, we found an increase in SOD activity, suggesting that O_2^- production is increased during vitamin A supplementation. We also observed that vitamin A supplementation decrease SOD immunocontent at high doses. SOD expression has been found to be low/absent in the fibrotic areas and pathological idiopathic pulmonary fibrosis (IPF) [34b]. Together, these results indicate a possible exacerbation in the O_2^- concentration in our experimental model. Then, increased O_2^- may favor SOD activity, because it is the major SOD allosteric activator [31].

An increase in SOD activity and a decrease in CAT activity occurring simultaneously may result in an increased concentration of hydrogen peroxide (H_2O_2), because its production from O_2^- by SOD is increased and its elimination by CAT is decreased. Then, an impaired SOD/CAT ratio is very likely to occur. H_2O_2 readily reacts with thiol functional groups, and this type of reaction is proposed to be a key mechanism by which ROS modulate cell signaling events.

Secondly, an excess of H_2O_2 facilitates the production of hydroxyl radical (OH), the most powerful oxidant molecule, through a reaction with iron or copper (Fenton chemistry) [31]. Thus, impaired SOD/CAT is very likely to culminate in increased oxidative damage to biomolecules. Therefore, the decrease in SOD or CAT immunocontent observed in this work probably is caused by the increased oxidative damage to biomolecules during vitamin A supplementation.

Increased protein carbonylation and excessive decrease in the protein thiol content may facilitate the formation of protein aggregates, as a result of protein cross-links, and this is very likely to culminate in widespread cellular dysfunctions. Additionally, increased oxidative damage to proteins might result in increased free iron, due to its release from damaged ferritin and other iron-containing proteins, favoring the maintenance of the pro-oxidative state by facilitates the production OH, the most powerful oxidant molecule, through a reaction with iron or copper (Fenton chemistry) [31, 35].

The increase in OH is also associated with increase in lipid peroxidation. We observed in this work that lipid peroxidation levels in the lungs of rats were increased. This effect was dose dependent. Products of lipid peroxidation are toxic; lipid hydroperoxides can directly inhibit enzymes. Moreover, increased oxidative damage may culminate in pathological conditions that lead to IPF [36].

In summary, our results show that vitamin A treatment increase oxidative stress parameters of rat lung. Unfortunately, it is almost impossible to indicate which vitamin A metabolite is the responsible for the observed effects, given the vast number of vitamin A metabolites existing [37–39]. Moreover, our research group has demonstrated the toxicity of vitamin A, at doses used in this work, also at the central nervous system [22–24]. However, the pro-oxidant effects here observed may be involved in the onset of lung diseases associated with redox dysfunctions and free radical–induced damage to biomolecules, such as preneoplasic transformation, lung fibrosis, and asthma, and more precautions should be taken when vitamin supplements are prescribed for therapeutic or preventive interventions. Therefore, these data suggest the importance of keeping vitamin A status within the physiological range, and reinforce the necessity of a better understanding of the redox properties and nonclassical actions of vitamin A and other retinoids on biological systems.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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CAPÍTULO II

"VITAMIN A SUPPLEMENTATION FOR DIFFERENT PERIODS ALTERS OXIDATIVE PARAMETERS IN LUNGS OF RATS"

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Full Communication

Vitamin A Supplementation for Different Periods Alters Oxidative Parameters in Lungs of Rats

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ABSTRACT   Lungs require an adequate supply of vitamin A (retinol) for normal embryonic development, postnatal maturation, and maintenance and repair during adult life. However, recent intervention studies revealed that supplementation with retinoids resulted in higher incidence of lung cancer, although the mechanisms underlying this effect are still unknown. Here, we studied the effect of vitamin A supplementation on oxidative stress parameters in lungs of Wistar rats. Vitamin A supplementation at either therapeutic (1,000 and 2,500 IU/kg) or excessive (4,500 and 9,000 IU/kg) doses for 3, 7, or 28 days induced lipid peroxidation, protein carbonylation, and oxidation of protein thiol groups, as well as change in catalase and superoxide dismutase activity. Together, these results suggest that vitamin A supplementation causes significant changes in redox balance, which are frequently associated with severe lung dysfunction.

KEY WORDS: • *lung* • *oxidative stress* • *retinol*

INTRODUCTION

IN THE PAST FEW YEARS, vitamin A (retinol) and its metabolites (retinoids) have been frequently suggested to be an important antioxidant for tissues such as lungs, liver, and heart.[1] Antioxidant activity has been reported for retinol as well as for many pro-vitamin A compounds, including β- and α-carotenes, by a mechanism of free radical scavenging and/or detoxification.[2] It has been demonstrated that retinoids inhibit cell growth and induce differentiation and apoptosis in a variety of normal and tumoral cells, thus acting on the prevention of the development of cancer from transformed cells.[3,4] In lungs, retinoids are essential for normal morphogenesis during the fetal period, for maturation and remodeling in the perinatal and postnatal periods, and for maintenance of the fully matured lungs.[5] However, clinical trials have demonstrated that the supplementation with retinoids may induce deleterious effects. Daily intervention with β-carotene (30 mg) combined with retinyl palmitate (25,000 IU/kg) increased the incidence of lung cancer and colorectal cancer in smokers and asbestos-exposed men.[5–9]

Oxidative stress is caused when the production of reactive oxygen species and/or reactive nitrogen species overcomes the cellular antioxidant defense systems. Oxidative stress cause structural changes and/or degradation of nucleic acids, proteins, and lipids. Exogenous antioxidants such as vitamin A contribute to prevent oxidative stress in many systems.[6] However, a growing body of evidence suggests that retinol and other retinoid derivatives may also exert pro-oxidant effects, which might lead to cell oxidative damage, transformation, and/or cell death.[10,11] Previously, we showed that retinol induces protein carbonylation and lipid peroxidation and that antioxidant enzyme activities are modulated by retinol[12–16] and *all-trans*-retinoic acid[12,15] treatments in cultured Sertoli cells. These pro-oxidant effects caused by retinol can lead to impaired cell signaling and induce malignant cell proliferation.[17–19] In addition, vitamin A directly induces overproduction of superoxide anion ($O_2^{-\bullet}$) *in vitro*, resulting in oxidative DNA damage.[20]

Increased oxidative stress is a significant part of the pathogenesis of lung cancer and is observed also in lung diseases such as asthma and chronic obstructive pulmonary disease and parenchymal lung diseases (*e.g.*, idiopathic pulmonary fibrosis and lung granulomatous diseases).[21] Reactive oxygen species/reactive nitrogen species may alter the remodeling of extracellular matrix, apoptosis, and mitochondrial respiration inside cells.[22] In addition, oxidative stress may impair the maintenance of surfactant and the antiprotease screen and the immune modulation in the lung.[22] Because oxidative stress is directly implicated on the pathogenesis of lung cancers and other pulmonary diseases[22,23] and because vitamin A supplementation is believed to induce pro-neoplasic effects, depending on the doses administered, we decided to investigate

the effects of vitamin A supplementation at either therapeutic (1,000 and 2,500 IU/kg) or excessive (4,500 and 9,000 IU/kg) doses on markers of oxidative stress in lungs of rats. Lipid peroxidation, protein carbonylation, protein thiol content, and antioxidant enzymes activities were assessed as markers of oxidative stress. We observed that vitamin A supplementation increases parameters of oxidative stress in lung at different doses, indicating the induction of oxidative stress in lungs.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (90 days old; weighing 280–320 g) were obtained from our own breeding colony. The animals were randomly separated into groups treated with vehicle (0.15 M saline; control group) or 1,000, 2,500, 4,500, or 9,000 IU/kg retinol palmitate (vitamin A). They were caged in groups of five animals with free access to standard commercial food (CR1 lab chow, Nuvilab, Curitiba, Brazil) and water and were maintained on a 12-hour light–dark cycle (7:00–19:00 hours) in a temperature-controlled colony room (23 ± 1°C). These conditions were maintained constant throughout the experiments. However, the number of rats in each group varied because of deaths that occurred during treatment, especially within groups treated with the higher concentrations of vitamin A. Therefore, the number of rats in each group varied between seven and 12 animals for group. All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health.[24] Our research protocol was approved by the Ethical Committee for Animal Experimentation of the Federal University of Rio Grande do Sul, Porte Alegre, RS, Brazil.

Drugs and reagents

Retinol palmitate (Arovit®, a commercial water-soluble form of vitamin A) was purchased from Roche (São Paulo, SP, Brazil). All other chemicals were purchased from Sigma (St. Louis, MO, USA). Vitamin A administration solutions were prepared daily and protected from light exposure and temperature.

Treatment

The animals were treated once a day for a period of 3, 7, or 28 days. All treatments were carried out at night (*i.e.*, when the animals are more active and eat a greater amount of food) in order to ensure maximum vitamin A absorption because this vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0.15 M saline) or 1,000, 2,500, 4,500, or 9,000 IU/kg retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.8 mL during each period of interest. Adequate measures were taken to minimize pain or discomfort.

Lung extraction and sample preparation

The animals were killed by decapitation 24 hours after the last vitamin A administration. The lung was dissected out on ice immediately after the rat was sacrificed and stored at −80°C for posterior biochemical analyses. The homogenates were centrifuged (700 g, 5 minutes) to remove cellular debris. Supernatants were used to all biochemical assays described. All the results were normalized by the protein content using bovine albumin as standard.[25]

Thiobarbituric acid-reactive species (TBARS)

As an index of lipid peroxidation, we measured the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described.[26] In brief, the samples were mixed with 0.6 mL of 10% trichloroacetic acid and 0.5 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 25 minutes. TBARS were determined by absorbance in a spectrophotometer at 532 nm. Results are expressed as nmol of TBARS/mg of protein.

Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine as previously described.[27] In brief, proteins were precipitated by addition of 20% trichloroacetic acid and redissolved in dinitrophenylhydrazine, and the absorbance was read in a spectrophotometer at 370 nm. Results are expressed as nmol of carbonyl/mg of protein.

Measurement of protein thiol content

Protein thiol content in samples was analyzed to estimate oxidative alterations in proteins. In brief, an aliquot was diluted in 0.1% sodium dodecyl sulfate, 0.01 M 5,5′-dithionitrobis(2-nitrobenzoic acid) in ethanol was added, and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 minutes.[28] Results are expressed as mmol of SH/mg of protein.

Estimation of antioxidant enzyme activities

Catalase (EC 1.11.1.6) (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm,[29] and the results are expressed as units of CAT/mg of protein. Bubble formation in oxygen generation by CAT activity was monitored and did not interfere with measurement of CAT activities in the linear range used to measure CAT activity. Superoxide dismutase (EC 1.15.1.1) (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described,[30] and the results are expressed as units of SOD/mg of protein. A ratio between SOD and CAT activities (SOD/CAT) was applied to better understand the effect of vitamin A supplementation on these two oxidant detoxifying enzymes that work in sequence converting superoxide anion to water.[31] An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

Statistical analyses

Both biochemical and behavioral results are expressed as means ± SEM values. *P* values were considered significant when *P* < .05. Differences in experimental groups were determined by one-way analysis of variance followed by the *post hoc* Tukey's test whenever necessary.

RESULTS

We observed an increased lipid peroxidation levels in the lungs of the rats that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3, 7, or 28 days (*P* < .05) F1 ▶ (Fig. 1A). This increase in lipid peroxidation levels in lungs of the rats that received vitamin A supplementation was dose dependent. Protein carbonylation levels increased (*P* < .05) in the lung of the rats treated with vitamin A at 2,500, 4,500, or 9,000 IU/kg for 3 or 7 days (Fig. 1B). Vitamin A supplementation increased lung carbonyl levels by twofold for 3 or 7 days. After 28 days of vitamin A supplementation any dose induced significant increase in the lung protein carbonylation levels (*P* < .05) (Fig. 1B). Vitamin A supplementation at any dose induced a significant decrease in the lung protein thiol content at 2,500, 4,500, or 9,000 IU/kg for 3 days (*P* < .05), and this decrease in protein thiol content was dose dependent (Fig. 1C). Interestingly, 7 days of vitamin A supplementation did not alter protein thiol levels. However, after 28 days of vitamin A supplementation protein thiol levels decreased at all doses (*P* < .05) (Fig. 1C), and this result was similar with the 3-day treatment.

We next investigated activities of antioxidant enzymes. SOD activity did not change in lungs of the rats that received F2 ▶ vitamin A supplementation for 3 or 7 days (Fig. 2A). However, SOD activity increased (*P* < .05) in lungs of the rats that received vitamin A supplementation at 4,500 or 9,000 IU/kg for 28 days (Fig. 2A). CAT activity did not change in lungs of the rats that received vitamin A supplementation for 3 or 7 days (Fig. 2B). In the rats supplemented for 28 days, however, all doses of vitamin A induced a significant decrease (*P* < .05) in lung CAT activity (Fig. 2B). Moreover, as shown in Figure 2C, vitamin A supplementation for 28 days at any dose tested induced an increase (*P* < .05) in lung SOD/CAT ratio. As a consequence of increased SOD/CAT ratio, H_2O_2 availability might be increased, favoring the permanence of a pro-oxidant state in lungs of the treated rats.

DISCUSSION

Vitamin A is widely suggested to be protective against oxidative stress.[1] Some authors have suggested that retinoids act as antioxidants and could be potential agents in antioxidant supplementation protocols for treatment and prevention of cancer and neurodegenerative diseases. In this context, clinical trials have been carried out based on the potential antioxidant role of vitamin A, but some of them had to be discontinued because of increased mortality related to lung cancer incidence.[6–9] It was suggested that
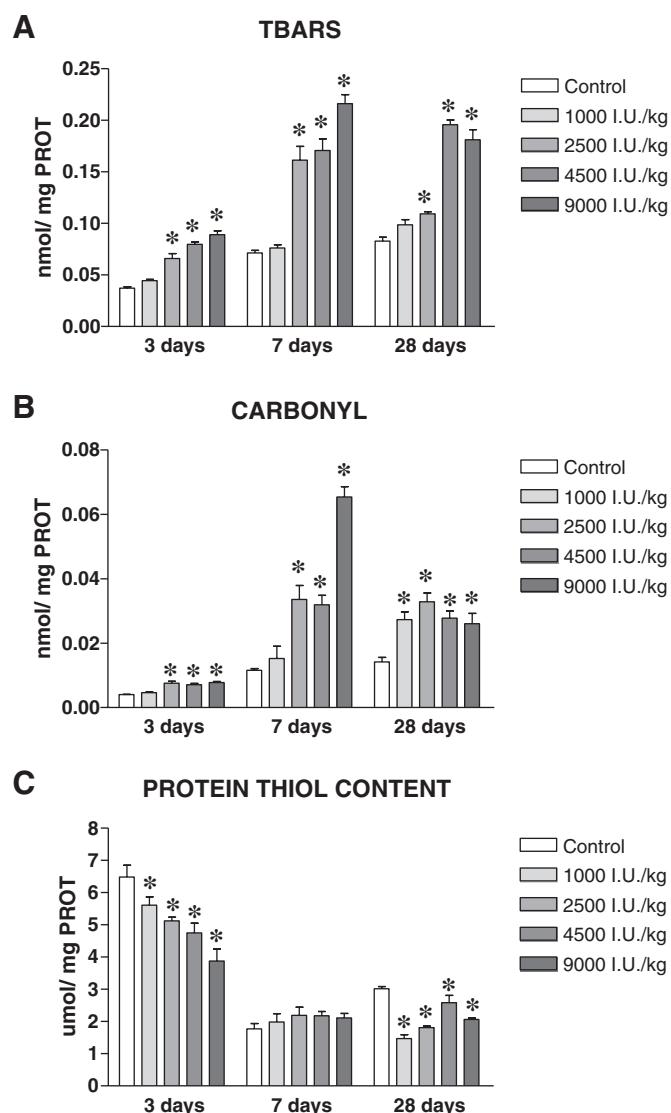
FIG. 1. Effects of vitamin A supplementation on (**A**) lipid peroxidation, (**B**) protein carbonylation, and (**C**) protein thiol content in rat lung. Data are mean ± SEM values (*n* = 7–12 per group). *P* < .05 (by one-way analysis of variance followed by the *post hoc* Tukey's test).

many of the deleterious effects observed in these studies could be related to free radical generation in vitamin A supplementation.[7] Thus, the redox properties of vitamin A and other retinoids may vary in biological systems, and the side effects resulting from preventive or therapeutic supplementations should not be neglected when considering the use of such protocols. We observed that vitamin A treatment at different doses or for different periods induced a pro-oxidant state in lungs of adult rats. Vitamin A treatment increased lipid peroxidation and protein carbonylation, but also decreased protein thiol content. Moreover, the activities of CAT and SOD were also modulated by vitamin A supplementation. It was observed that in hypervitaminosis A, the levels of plasma and tissue retinol do not correlate with

A

SOD

B

CATALASE

C
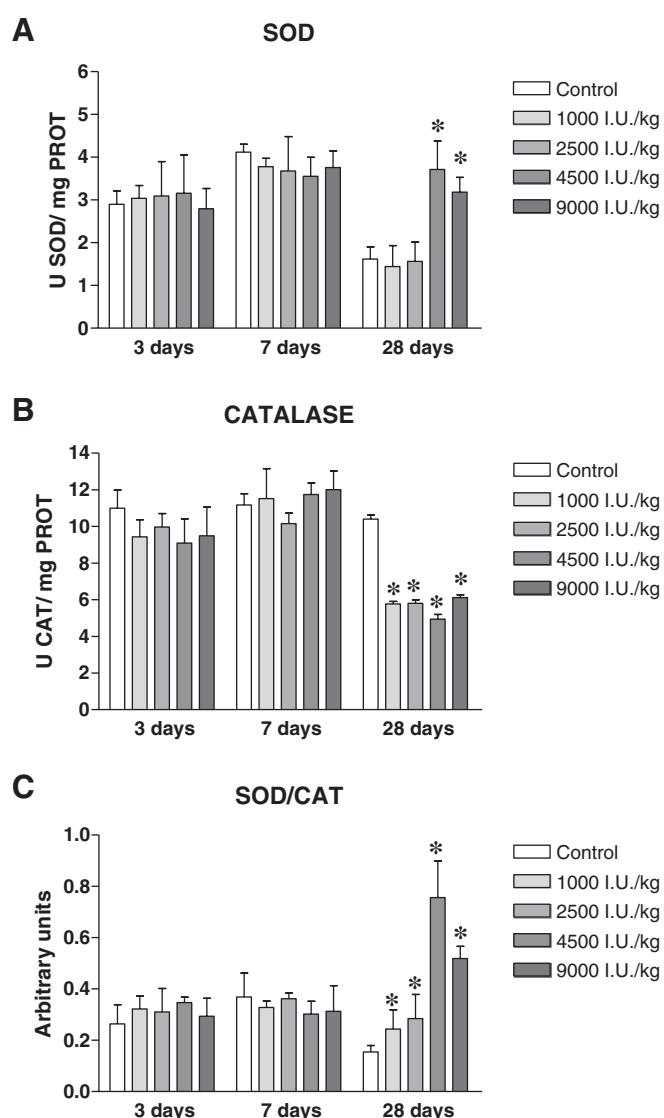
SOD/CAT

FIG. 2.   Effects of vitamin A supplementation on (**A**) SOD and (**B**) CAT activity in the rat lungs. (**C**) Lung SOD/CAT ratio. Data are mean $\pm$ SEM values ($n = 7$–12 per group). *$P < .05$ (by one-way analysis of variance followed by the *post hoc* Tukey's test).

the increased intake,[32–35] suggesting that retinol is converted to several other metabolites when increasing doses are ingested.[32–36]

Unfortunately, it is almost impossible to determine which vitamin A metabolite is the responsible for the pro-oxidant effects observed in our model, given the vast number of vitamin A metabolites existing, but it is possible that retinol is not the only effector. Van Helden *et al.*[37] showed that both β-carotene and its metabolites (vitamin A, retinal, and retinoic acid) were able to increase hydroxyl radical ($OH^\bullet$) formation from a system containing iron and hydrogen peroxide (H_2O_2) through the Fenton reaction.

It is known that vitamin A autooxidation *in vitro* increases superoxide anion ($O_2^{-\bullet}$) production, and this was also observed in cell cultures.[20,38] Previous studies showed that

vitamin A leads to an impairment on electron transfer system, thus increasing the rate of $O_2^{-\bullet}$ production. This effect was reported either in liver isolated mitochondria incubated with retinol or in mitochondria isolated from vitamin A-supplemented rats.[37–39] $O_2^{-\bullet}$ is a well-known CAT inhibitor,[40,41] and we observed that vitamin A supplementation for 28 days decreases CAT activity. Moreover, we found an increase in SOD activity, suggesting that $O_2^{-\bullet}$ production is increased during vitamin A supplementation. Together, these results indicate a possible exacerbation in the $O_2^{-\bullet}$ concentration in this experimental model. Increased $O_2^{-\bullet}$ may favor SOD activity because it is the major SOD allosteric activator.[30] Then, an impaired SOD/CAT ratio is very likely to occur.

An increased SOD activity and decreased CAT activity occurring simultaneously may result in an increased concentration of H_2O_2 because its production from $O_2^{-\bullet}$ by SOD is enhanced and its elimination by CAT is decreased. Second, an excess of H_2O_2 facilitates the production of $OH^\cdot$, the most powerful oxidant molecule, through a reaction with iron or copper (Fenton chemistry).[31] *In vitro* experiments show that either β-carotene or retinol is able to increase iron uptake,[14,36,41] making more iron available in the cells to react with H_2O_2. In addition, H_2O_2 is relatively stable and able to diffuse into the nucleus, where it can react with DNA-associated transition metals to form $OH^\bullet$. Oxidatively damaged DNA is known to be pro-mutagenic and is therefore an important marker for carcinogenesis. Previously, we showed that retinol increases iron uptake and induces damage and mutagenesis in DNA of Sertoli cells through iron-dependent Fenton chemistry.[14,36] Other authors also observed that vitamin A was able to induce damage in DNA.[20,36]

An imbalance between SOD/CAT ratio is thought to facilitate oxidative-dependent alterations in the cellular environment because of accumulation of H_2O_2 and consequent availability of this compound to react with iron to form $OH^\bullet$; thus impaired SOD/CAT is very likely to culminate in increased oxidative damage to biomolecules. The basal or physiological SOD/CAT ratio varies according to tissues, depending on their basal production of superoxide and H_2O_2 from electron transport chain, peroxisomes, and other oxidative processes. Many authors have shown that an imbalance between SOD/CAT is responsible for increasing oxidative stress in biological systems.[39–41] Our results showed that vitamin A supplementation for 28 days increases this ratio in lungs of treated rats, which indicates that SOD and CAT detoxifications of superoxide and H_2O_2 were uncoupled. This imbalance may be responsible for increasing in oxidative damage in lung biomolecules. It must be taken into account that rats treated for different periods with vitamin A have different ages. Therefore, the results presented here were always compared with the control group of rats within the same period of treatment, and the differences observed between control groups of rats from different ages were expected. It is well known that expression and activity of antioxidant enzymes vary according to aging, generally decreasing the ability of cells to clear free

radical production,[36] but the aim of the present work was not to explore such age-dependent differences; instead, we focused on different periods of supplementation compared to their relative controls.

A large portion of biological properties and functions involving protein structure, enzyme catalysis, and redox-signaling pathways depends on the redox properties of the thiol group present both in protein and in low-molecular-weight molecules. Numerous cell signaling proteins are regulated by alterations of the protein thiol groups.[42] This signaling involves cell proliferation, differentiation, and death. Increased protein carbonylation and excessive decrease in the protein thiol content may facilitate the formation of protein aggregates, as a result of protein cross-links, and this is very likely to culminate in a widespread cellular dysfunction. Additionally, increased oxidative damage to proteins might result in increased free iron, because of its release from damaged ferritin and other iron-containing proteins, favoring the maintenance of the pro-oxidative state.[43–47] We observed that vitamin A supplementation increases protein carbonylation in all periods analyzed. In addition, protein thiol levels were decreased with vitamin A supplementation for 3 or 28 days, but were not different between treatments in 7 days. These effects are also observed in pathological conditions that lead to idiopathic pulmonary fibrosis or lung cancer.[21] Interestingly, there was a significant difference in protein thiol content between control animals in the different periods of treatment. These alterations in protein thiol level could be age-dependent.

In summary, our results show that vitamin A treatment increases oxidative stress parameters of rat lungs. Furthermore, these pro-oxidant effects here observed may be involved in the onset of lung diseases associated to redox dysfunctions and free radical-induced damage to biomolecules, such as lung cancer, lung fibrosis, and asthma, and more precautions should be taken when vitamin supplements are prescribed for therapeutic or preventive interventions.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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PARTE III

4.DISCUSSÃO

A vitamina A é amplamente sugerida como um agente antioxidante, o qual protegeria os sistemas biológicos contra processos de estresse oxidativo. Isto se deve ao fato dela ser considerada por alguns autores como um *scavenger* de radicais livres. Por esse motivo o tratamento/suplementação com retinóides é amplamente sugerido em protocolos para prevenção ou tratamento de câncer e doenças neurodegenerativas. No entanto, neste contexto, demonstrou-se que a suplementação alimentar de vitamina A aumenta a incidência de câncer de pulmão e problemas cardiovasculares em populações sujeitas a certos fatores de risco(por exemplo, fumantes e trabalhadores de minas de asbesto) e, por esses motivos os estudos tiveram que ser interrompidos devido ao aumento da mortalidade relatada. Os autores sugeriram que os efeitos observados nesses estudos poderiam estar relacionados a geração de radicais livres pela suplementação com vitamina A.

Neste trabalho, mostramos que suplementação com palmitato de retinol nos períodos de 3, 7 ou 28 dias, em doses terapêuticas (1000 ou 2500 UI/kg/dia) e supra-terapeuticas (4500 ou 9000 UI/kg/dia) foram capazes de alterar parâmetros de estresse oxidativo nos pulmões de ratos machos adultos. Além disso, não foi observada qualquer adaptação ao insulto oxidativo induzido, já que os marcadores de peroxidação lipídica, de carbonilação de proteínas e de estado redox de grupamentos tióis não retornaram a valores de controle mesmo após 28 dias de tratamento. Aumento nos níveis de carbonilação de proteínas e de peroxidação lipídica e uma diminuição na concentração de grupamentos tióis reduzido foram observado. O tratamento modificou a atividade de enzimas antioxidantes tais como SOD e CAT. O imunoconteúdo dessas enzimas também se mostrou alterado

com o tratamento. A alteração nas atividades de SOD e de CAT, assim como no imunoconteúdo, levaram a um desequilíbrio na relação SOD/CAT, pois, o tratamento com palmitato de retinol induziu aumento na atividade da SOD e diminuição na atividade da CAT, o que favorece um aumento na concentração de H_2O_2 no seu sítio de produção, que pode ser mitocondrial (Mn-SOD) ou citosólico(Cu/Zn-SOD). O excesso de H_2O_2 facilita a produção de $OH^\bullet$, o mais instável dentre os radicais livres, ou seja, é aquele que tem o maior potencial de causar dano a biomoléculas, seja por redução ou por oxidação destas.

Infelizmente, é impossível determinar qual metabólito de vitamina A é responsável por esse efeito pró-oxidante observado em nosso modelo, tendo em vista o grande número de metabólitos que a vitamina A pode derivar. Van Helden *et. al.* demonstraram que, tanto o β-caroteno como seus metabolitos (vitamina A, retinal, ácido retinóico), são capazes de aumentar a formação do radical $OH^\bullet$ em sistemas contendo ferro e H_2O_2 através da reação de Fenton.Também é conhecido que a auto-oxidação da vitamina A *in vitro* aumenta a produção do anion $O_2^{\bullet-}$, e o mesmo já foi observado pelo nosso grupo de pesquisa em modelo de cultura celular. Estudos anteriores também relatam que a vitamina A pode prejudicar o sistema de transferência de elétrons na cadeia transportadora de elétrons, então aumentando a taxa de produção de $O_2^{\bullet-}$. Este efeito foi relatado tanto em mitocôndrias isoladas de fígado e tratadas com retinol quanto com mitocôndrias isoladas de fígados de ratos suplementados com vitamina A.

É bem conhecido que $O_2^{\bullet-}$ é um inibidor da CAT. Em nosso trabalho, foi possível observar que a suplementação com vitamina A por 28 dias diminui a atividade da CAT em 50% quando comparada com a atividade do controle. Além disso, nós encontramos um aumento na atividade de SOD nesse mesmo período, sugerindo que a formação de $O_2^{\bullet-}$ esteja aumentada durante o tratamento com vitamina A. Juntos, esses resultados indicam um possível aumento na concentração de $O_2^{\bullet-}$ nesse modelo experimental.

Este provável aumento de $O_2^{\bullet-}$ pode favorecer a atividade de SOD, isto porque ele é o seu principal ativador alostérico. Essa modulação na atividade de SOD e CAT, observado em nosso modelo, pode levar a um desequilíbrio na relação SOD/CAT. Um aumento da atividade de SOD ocorrendo simultaneamente com diminuição da atividade de CAT pode resultar em um aumento na concentração de H_2O_2, isto devido a sua produção a partir $O_2^{\bullet-}$ através da atividade de SOD estar aumentada e sua eliminação pela CAT estar diminuída. Experimentos *in vitro* já demonstraram que tanto β-caroteno quanto retinol são capazes de aumentar a captação de ferro e disponibilidade para as células, dessa maneira favorecendo uma possível reação de Fenton. Além disso, H_2O_2 é relativamente estável e capaz de se difundir para núcleo, onde pode vir a reagir com metais de transição associados ao DNA e formar então o radical $OH^{\bullet}$. O

dano oxidativo a DNA é conhecido como sendo mutagênico e, portanto um importante marcador mutagênico.

Essa alteração que um desbalanço na relação SOD/CAT causa pode levar a produção de espécies reativas do oxigênio (EROs) e de espécies reativas do nitrogênio (ERNs), as quais podem atacar proteínas, danificando as mesmas levando à formação de proteínas carboniladas. O aumento dos níveis de carbonilação de proteínas reflete os níveis de oxidação protéica nas células. A carbonilação de proteínas pode ser gerada através da quebra de estrutura da proteína, retirada de um átomo de hidrogênio no carbono alfa, ataque há diversos aminoácidos de cadeia lateral (como lisina, arginina, prolina, etc.), pela formação dos adutos de Michael entre os resíduos de lisina, histidina e cisteína, e através da ação de produtos reativos da peroxidação lipídica. A glicação e glicoxidação nos grupos amino de Lisina pode levar a formação dos chamados produtos finais avançados de glicação (AGEs), os quais também podem levar à carbonilação de proteína. A geração de radicais protéicos, então, pode levar à formação de outros radicais, os quais podem causar danos a outras biomoléculas, gerando então uma cadeia de reações de oxidação/redução. Muitos danos a proteínas são irreparáveis e podem levar a conseqüências como dimerização ou agregação, desdobramento ou mudanças conformacionais, expondo resíduos mais hidrofóbicos ao ambiente aquoso celular, perda de estrutura ou atividade funcional, alteração na renovação, modulação na regulação gênica tanto a nível de expressão quanto de regulação, modulação da sinalização celular, podendo levar a indução de apoptose ou necrose. Portanto, os níveis de oxidação protéica tem significância fisiológica e patológica. Por esse motivo, a identificação de proteínas carboniladas serve como

parâmetro para ver alterações nas proteínas, quer seja à proteína estrutural ou enzimática.

Como as propriedades biológicas, na sua maior parte, envolvem a estrutura de proteínas, catálise enzimática e vias de sinalização redox sensíveis, as quais dependem das propriedades dos grupamentos tióis presentes tanto em proteínas quanto em moléculas de baixo peso molecular, diversas vias de sinalização celular são reguladas através de alterações no estado de oxidação de grupamentos tióis. Essa sinalização participa de processos como proliferação celular, diferenciação celular e morte celular. Portanto, qualquer alteração que venha a modular o estado redox dos grupamentos tióis pode vir a modular o ciclo da célula na qual essa modificação ocorreu. Além disso, um aumento de carbonilação de proteínas juntamente com alteração redox nos grupamentos tióis pode facilitar a formação de agregados protéicos, pelo fato de favorecer ligações entre as próprias proteínas que estão danificadas, o que então pode acarretar uma disfunção celular. O aumento de dano oxidativo a proteínas também pode levar a um aumento de ferro livre, isso porque ele pode ser liberado a partir de ferritina danificada ou outras proteínas que contenham ferro, dessa maneira favorecendo a manutenção de um estado pró-oxidativo. Nós observamos que a suplementação com vitamina A aumentou os níveis de carbonilação de proteína em todos os períodos analisados. Os níveis de grupamentos tióis protéicos também se alteraram com a suplementação com vitamina A, diminuindo seus níveis nos períodos de 3 e 28 dias. Entretanto, no período de 7 dias, os níveis não sofreram nenhuma alteração. Todos os efeitos observados aqui neste trabalho são também efeitos encontrados em condições patológicas como fibrose pulmonar e câncer de pulmão.

Então, com base nos resultados apresentados, mostramos que a vitamina A, em doses que antes eram tidas como seguras ao organismo humano, induziram estresse oxidativo no pulmão de ratos adultos. Doses elevadas de vitamina A e de alguns de seus retinóides(como o ácido retinóico 13-*cis*) são utilizadas terapeuticamente por períodos muito variados, desde dias até meses. Como conseqüências dos tratamentos crônicos, já foram encontrados efeitos colaterais envolvendo a função cerebral humana em adultos, tais como irritabilidade, depressão e ansiedade. Entretanto, os efeitos de uma suplementação com vitamina A em parâmetros de estresse oxidativo em pulmões nunca haviam sido observados. Baseados em nossos resultados, acreditamos que futuros estudos podem mostrar a possível correlação entre a administração de palmitato de retinol e disfunções pulmonares.

5.CONCLUSÕES

A partir dos resultados obtidos no presente trabalho, podemos concluir que:

1- A suplementação com vitamina A, tanto nas doses consideradas terapêuticas quanto naquelas supra-terapêuticas – geralmente atingidas em tratamentos prolongados, ou devido ao uso inadvertido da vitamina, é capaz de induzir, no pulmão de ratos, aumento nos marcadores de estresse oxidativo mesmo em períodos agudos de tratamento – 3 dias. Além disso, não foi observada qualquer adaptação ao insulto oxidativo induzido, já que os marcadores de peroxidação lipídica, de carbonilação de proteínas e de estado redox de grupamentos tióis não retornaram a valores de controle mesmo após 28 dias de tratamento;

2- O tratamento foi capaz de modular a atividade de enzimas antioxidantes nos pulmões analisados, com um aumento marcante na atividade de SOD e uma diminuição na atividade de CAT no período de 28 dias. Além disso, o imunoconteúdo de SOD assim como o de CAT encontraram-se reduzidos nas maiores doses utilizadas para suplementação em 28 dias. Tais resultados podem indicar um aumento na produção de $O_2^{\bullet-}$, com conseqüente metabolização em H_2O_2 pela SOD, que parece não ser

reduzida a água em taxas normais devido à baixa atividade de CAT. Assim, uma facilitação na reação de Fenton pode ser esperada, onde os íons como Fe^{2+} ou Cu^{2+}, ao reagirem com o H_2O_2, podem originar o radical $OH^{\bullet}$,o mais deletério dos radicais livres;

3- Em suma, mostramos que a suplementação com vitamina A na forma hidrossolúvel (palmitato de retinol - Arovit®) foi capaz de induzir um estado pró-oxidante nos pulmões de ratos. É importante salientar que não houve adaptação ao dano induzido, pois, em alguns casos, o dano oxidativo encontrado após 3 dias de suplementação é muito semelhante ao observado após 28 dias de suplementação. Também é interessante mencionar que o motivo que favorece o dano agudo nem sempre é o mesmo que favorece aquele crônico, ou seja, a disfunção observada cronicamente pode ser resultado de uma perturbação à homeostasia de organelas celulares, por exemplo.

6.PERSPECTIVAS

Os resultados obtidos no presente trabalho abrem espaço para pesquisas mais apuradas no que diz respeito ao estado redox dos pulmões. Como principais perspectivas de continuação deste trabalho, temos:

1- Analisar se a suplementação com vitamina A é capaz de alterar os níveis do receptor para produtos avançados finais de glicação (RAGE), visto que a modulação desse receptor pode levar a alteração no destino celular (apoptose ou proliferação) através da modulação do fator de transcrição NF-κB;

2- Investigar o funcionamento da cadeia transportadora de elétrons das células pulmonares para melhor compreensão dos efeitos gerados através da suplementação com vitamina A;

3- Analisar o lavado bronco alveolar de ratos suplementados, para verificar se a suplementação com palmitato de retinol pode favorecer a um estado pró-inflamatórios nos pulmões desses animais.

7. ANEXO

Segue em anexo artigo publicado durante o período de mestrado.
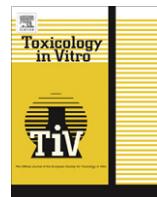
Retinol and retinoic acid modulate catalase activity in Sertoli cells by distinct and gene expression-independent mechanisms

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ABSTRACT

Vitamin A (retinol) exerts a major role in several biological functions. However, it was observed that retinol induces oxidative stress on different cellular types. Catalase (EC 1.11.1.6; CAT) is a hydrogen peroxide metabolizing enzyme, and its activity and expression is widely used as an index to measure oxidative stress and perturbations in the cellular redox state. The aim of this study was to investigate the effects of retinol and its major biologically active metabolite, all-trans retinoic acid (RA), on CAT regulation. For this purpose, cultured Sertoli cells (a physiological target of vitamin A) were treated with retinol or RA. Retinol (7 μM, 14 μM) and RA (100 nM, 1 μM) enhanced intracellular reactive species production and increased CAT activity after 24 h of treatment. Retinol increased CAT immunocontent but did not alter CAT mRNA expression, while the increase in CAT activity by RA was not related to alterations in immunocontent or mRNA expression. In vitro incubation of purified CAT with retinol or RA did not alter enzyme activity.

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1. Introduction

Vitamin A (retinol) and its metabolites are essential micronutrients that participate in processes such as vision, immunity, reproduction, growth, and development (Olson, 1984; Blomhoff, 1994). Retinol is converted into retinoic acid (RA) by multi-step enzymatic reactions inside cells, thus activating the nuclear retinoid receptors. These receptors are subdivided into retinoid acid receptors (RAR) and retinoid X receptors (RXR), and it is generally accepted that most of biological actions of retinol arises from the RA-mediated RAR/RXR activation (Mangelsdorf et al., 1994).

Both retinol and RA have been suggested as potential therapeutic agents in the treatment of a wide range of diseases, including leukemia, thyroid cancer, and xeroderma pigmentosum (Altucci and Gronemeyer, 2001). Besides, vitamin A supplementation has been demonstrated to be effective as an antioxidant (Burton and Ingold, 1984). However, some authors observed that vitamin A may induce toxic effects to different cell types (Bendich and Langseth, 1989; Hathcock et al., 1990), and it has been recently suggested that toxic effects of retinol and other retinoid derivatives are related to pro-oxidant properties (Polyakov et al., 2001; Mayne et al., 1991).

Hydrogen peroxide (H_2O_2) participates in the cellular homeostasis as a signal transduction regulator, since it is readily diffusible across membranes (Veal et al., 2007). However, excessive H_2O_2 production is deleterious to many cellular functions (Davies et al., 1987). Catalase (EC 1.11.1.6; CAT) is one of the main H_2O_2 metabolizing enzymes, converting H_2O_2 into H_2O and O_2. CAT activity is closely associated to cellular proliferative activity in many normal and transformed cell types (Komuro et al., 2005; Hachiya and Akashi, 2005; Brown et al., 1999), and its regulation is a major mechanism of defense against cytotoxic effects of H_2O_2.

We have previously observed that, under certain conditions, both retinol and RA are able to induce a pro-oxidant state in cultured Sertoli cells, leading to increased lipoperoxidation, protein carbonylation and DNA oxidative damage (Dal-Pizzol et al., 2001a, 2000; Frota et al., 2006). These effects are accompanied by the activation of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Dal-Pizzol et al., 2001a). Besides, we observed that retinol and RA led to an increase in CAT activity after a 24 h period of incubation, suggesting that vitamin A modulates the expression or activity of this enzyme.

In this work, we investigated how vitamin A (i.e., retinol and its most biologically active metabolite, RA) increases CAT activity in Sertoli cells, and whether this activation is caused by retinol itself or by its conversion into RA. For this purpose, we incubated Sertoli cells with retinol or RA and analyzed intracellular reactive species

51

formation, CAT activity, CAT immunocontent, and CAT mRNA expression. Our results indicate that retinol and RA caused different profiles of reactive species formation in Sertoli cells, which led to different mechanisms of CAT activation.

2. Materials and methods

2.1. Materials and animals

All drugs were purchased from Sigma Chemicals (St. Louis, MO, United States). All-*trans*-retinol and all-*trans* retinoic acid (RA) were dissolved in ethanol. Concentrated stocks were prepared immediately before experiments by diluting retinol or RA into ethanol and determining final stock concentration by UV absorption; solution was kept protected from light and temperature during all procedures. Appropriate solvent controls were performed for each condition. The final ethanol concentration did not exceed 0.2% in any experiment. CAT purified protein was purchased from Sigma Chemicals (St. Louis, MO, United States). Anti-CAT (catalase) was purchased from Calbiochem (San Diego, CA, United States). Anti-β-actin was purchased from Sigma Chemicals (St. Louis, MO, United States). Horseradish peroxidase-coupled anti-IgG antibody was from Amersham Pharmacia Biotech (Piscataway, NJ, United States). The West Pico chemiluminescent Kit was obtained from Pierce (Rockford, IL, United States). Pregnant Wistar rats were housed individually in plexiglass cages. Litters were restricted to eight pups each. Animals were maintained on a 12-h light/dark cycle at a constant temperature of 23 °C, with free access to commercial food and water. Male immature rats (15 days old) were killed by cervical dislocation.

2.2. Isolation and culture of Sertoli cells

Sertoli cells were isolated as previously described (Moreira et al., 1997). Briefly, testes of 15-day-old rats were removed, decapsulated and digested enzymatically with trypsin and deoxyribonuclease for 30 min at 34 °C, and centrifuged at 750g for 5 min. The pellet was mixed soybean trypsin inhibitor, then centrifuged and incubated with collagenase and hyaluronidase for 30 min at 34 °C. After incubation, this fraction was centrifuged (10 min at 40g). The pellet was taken to isolate Sertoli cells and supernatant was discarded. After counting, Sertoli cells were plated in 6 x dishes multiwell plates (3×10^5 cells/cm^2) in Medium 199, pH 7.4, 1% FBS, and maintained in humidified 5% CO$_2$ atmosphere at 34 °C for 24 h for attachment. The medium was then changed to serum-free medium and cells were maintained for more 24 h. Sertoli cells cultures were estimated to be 90–95% pure, as assessed by the alkaline phosphatase assay.

2.3. Catalase enzyme activity

Enzyme assay were performed in cells extracts obtained as follows. Cells were harvested and washed three times with saline. To determine CAT activity cells were sonicated in 50 mM phosphate buffer (pH 7.0) and the resulting suspension as centrifuged at 780g for 10 min. The supernatant was used for enzyme assays. CAT activity was assayed by measuring the rate of decrease in H$_2$O$_2$ absorbance at 240 nm (Aebi, 1984).

2.4. Determination of intracellular reactive species

Intracellular reactive species production was determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, 1×10^5 cells were plated on 96-well plates and 100 μM DCFH-DA dissolved in medium containing 1% FBS was added to each well 30 min before stop treatment to allow cellular incorporation. This incubation medium was then replaced to fresh medium and DCFH-DA oxidation was monitored by 30 min with 5 min intervals at 34 °C in a fluorescence plate reader (F2000, Hitachi Ltd., Tokyo, Japan) with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm. The percentage increase in each well was calculated according to the formula $[(Ft_{30} - Ft_0)/Ft_0 \times 100]$, where Ft_{30} = fluorescence at time 30 min and Ft_0 = fluorescence at time 0 min. This method of analysis has advantages over analyzing just the net change in fluorescence in that, not only did the calculated data directly reflect the percentage changes of fluorescence over time from the cells in the same well, they also effectively control for variability among wells. This method also canceled out the background fluorescence in each well, and therefore, a "no cell" control is not needed (Wang and Joseph, 1999).

2.5. Immunoblot

To perform immunoblot experiments, Sertoli cells cultures were lysed in Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) and equal amounts of cell protein (30 μg/well) were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinyledilene difluorife (PVDF) membranes. Protein loading and electroblotting efficiency were verified through Ponceau S staining, and the membrane was blocked in Tween–Tris buffered saline (TTBS: 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin and incubated overnight with the primary antibody to be tested. The membrane was washed again and the immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis of the films was performed with the IMAGE J® software. Blots were developed to be linear in the range used for densitometry. All results were expressed as a relative ratio between the CAT immunocontent and the β-actin internal control immunocontent.

2.6. RNA extraction, cDNA and real-time PCR

RNA was isolated from 1 to 2×10^6 Sertoli cells using the Trizol Reagent (Invitrogen, Carlsbad, CA, United States). Approximately 1 μg of total RNA was added to each cDNA synthesis reaction using the SuperScript-II RT pre-amplification system (Invitrogen). Reactions were performed at 40 °C for 1 h using the primer T23 V (50 TTT TTT TTT TTT TTT TTT TTT TTV). Real-time PCR amplification was carried out using specific primer pairs designed with Oligo Calculator version 3.02 (http://www.basic.nwu.edu/biotools/oligocalc.html) and synthesized by RW-Genes. Mouse catalase (CAT) and β-actin gene specific primers were as follows: CATF (50-ACATGGTCTGGGACTTCTGG), CATR (50-CAAGTTTTTGATGCCCTGGT), bACTF (50-TATGCCA ACACAGTGCTGTCTGG) and bACTR (50-TACT-CCTGCTT GCTG ATCCA CAT). Real-time PCRs were carried out in an Applied-Biosystem 7500 real-time cycler (Applied-Biosystems, Foster City, CA, United States). Reaction settings were composed of an initial denaturation step of 5 min at 94 °C followed by 45 cycles of 10 s at 94 °C, 15 s at 60 °C, and 15 s at 72 °C; samples kept for 2 min at 40 °C for reannealing and then heated from 55 to 99 °C with a ramp of 1 °C/s to acquire data to produce the denaturing curve of the amplified products. Real-time PCRs were made in 10 μl final volume composed of 5 μl of each reverse transcription sample diluted 50 times, 1 μl of Platinum Taq 10 times PCR buffer, 0.6 μl of 50 mM MgCl$_2$, 0.2 μl of 5 mM dNTPs, 0.2 μl of 10 μM primer pairs, 2.45 μl of water, 0.5 μl of SYBR (1:100 000 Molecular Probe), and 0.05 μl of Platinum Taq DNA polymerase (5 U/μl) (Invitrogen). All results were expressed as a relative ratio between the CAT gene and the β-actin internal control gene.

52

2.7. "In vitro" CAT activity assay

The sample containing purified CAT from bovine liver (1 mg/mL), purchased from Sigma Chemicals (Catalogue C9322, St. Louis, MO, United States), were incubated in 50 mM phosphate buffer (pH 7.0) at 25 °C in the dark for 1, 12, or 24 h with retinol or RA in the presence of absence of Trolox® (100 μM). CAT activity was assayed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm (Aebi, 1984).

2.8. Protein quantification

Protein content of each sample was quantified (Lowry et al., 1951) for data normalization.

2.9. Statistical analysis

Data are expressed as means ± SEM and analyzed by one-way ANOVA followed by Tukey's post hoc test. Differences were considered to be significant at $p < 0.05$ level.

3. Results

In previous works, we observed that 24 h incubation with retinol or RA induced cell oxidative damage and increased CAT activity in Sertoli cells, suggesting that these compounds induce an unbalance in the cell redox state (Dal-Pizzol et al., 2001a; Frota et al., 2006). Thus, we evaluated here the ability of retinol and RA to induce reactive species production in Sertoli cells. For this purpose, Sertoli cells were incubated with retinol or RA for 24 h and intracellular reactive species formation was measured by the DCFH-DA assay. A range varying from physiological to supra-

physiological and pharmacological concentrations of retinol and RA were examined at an early time point (1 h), an intermediary stage (12 h) and then at 24 h of treatment, when most effects of retinol and RA on antioxidant enzymes were previously reported (Dal-Pizzol et al., 2001a,b; Klamt et al., 2003; de Oliveira et al., 2005; Frota et al., 2006).

Retinol at 7 μM and 14 μM significantly increased DCF fluorescence at 1 h (41% and 61%, respectively, compared to 7% increase in control cells – Fig. 1A). This effect was further sustained at 12 and 24 h, when retinol at 7 μM caused the greatest increase in intracellular reactive species formation (65%, compared to 30% of fluorescence increase in control cells). RA had a different profile; at 1 h, all concentrations decreased basal DCF fluorescence, except RA 1 μM (Fig. 1B, insert). At longer periods, however, RA at 100 nM and 1 μM both significantly increased intracellular reactive species formation (50% and 41%, respectively, compared to 30% in control cells – Fig. 1B). Antioxidant co-treatment with Trolox 100 μM inhibited the production reactive species induced by pro-oxidant concentrations of retinol and RA (Fig. 1A and B).

Next, we measured CAT activity in cells incubated with different concentrations of retinol and RA for the same time periods depicted above. After 1 h, CAT activation was only observed with retinol 5 μM (Fig. 2A). At longer periods, however, retinol 7 μM was more effective, and this concentration caused the most pronounced activation on CAT after 24 h (155%). In cells treated with RA, there was an increase in enzyme activity at 12 h, and further enhanced CAT activity at 24 h (Fig. 2B). We also evaluated the effect of antioxidant co-treatment on CAT activation by retinol and RA. As expected, Trolox reversed the increase in CAT activity induced by retinol, but, surprisingly, had no effect on CAT activation by RA (Fig. 2A and B), suggesting that retinol and RA modulate CAT by different mechanisms.
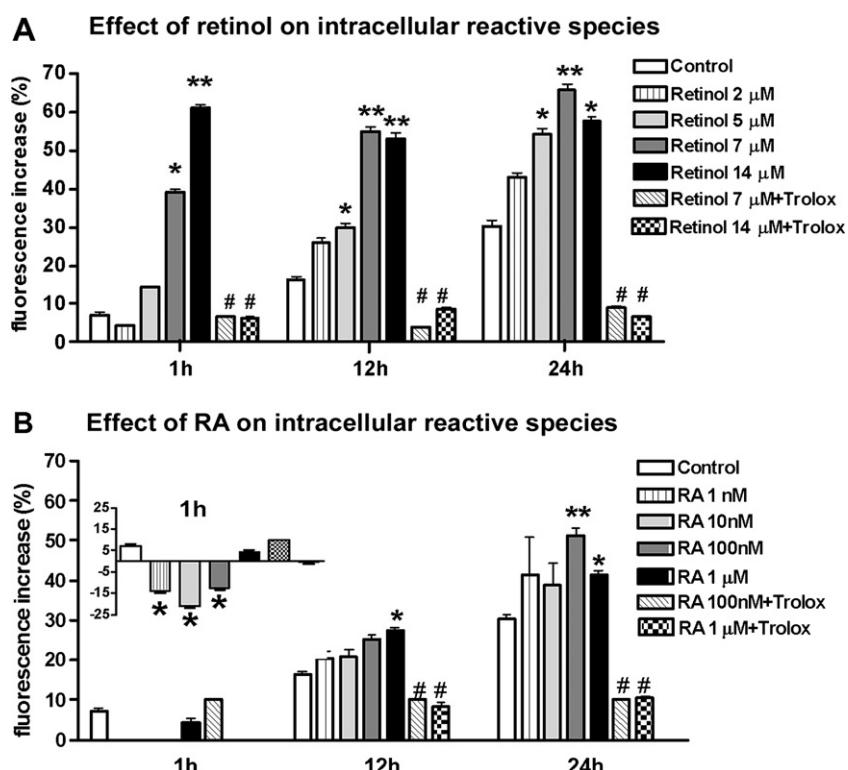
Fig. 1. Intracellular reactive species production by the DCFH-DA assay. Sertoli cells were treated with varying concentrations of retinol (A) and RA (B) for 1, 12 and 24 h, and the intracellular reactive species production was evaluated by the DCFH-DA assay, as depicted in Section 2. The effect of the antioxidant Trolox (100 μM) was evaluated with retinol at 7 and 14 μM (A) and RA at 100 nM and 1 μM (B). Values represent means ± SEM of four independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, compared to control; $^\#p < 0.05$, compared to * and ** groups, as analyzed by ANOVA (Tukey's post hoc).
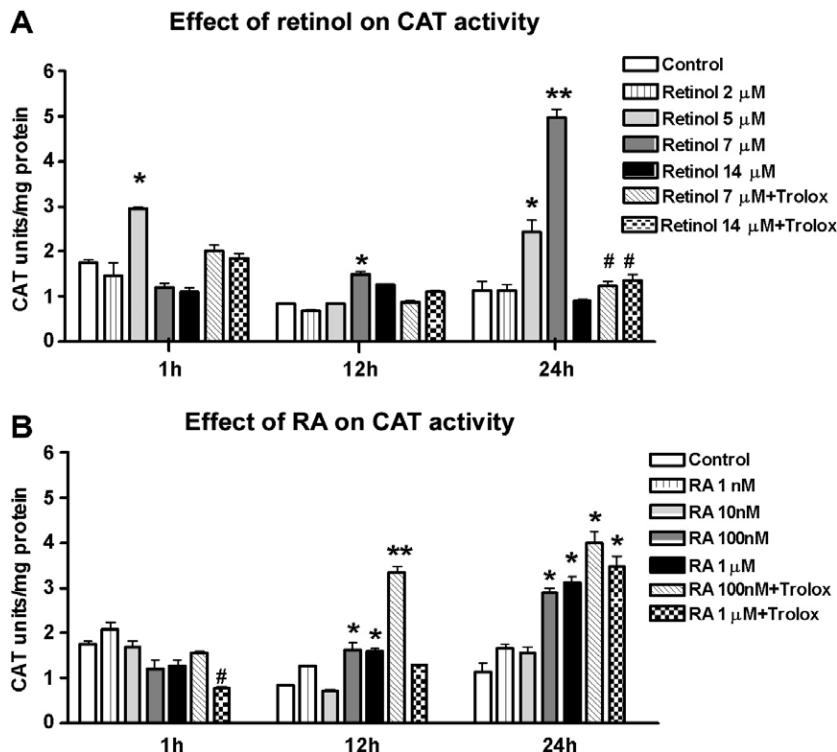
Fig. 2. CAT activity in Sertoli cells treated with retinol and RA. Sertoli cells were treated for 1, 12 and 24 h with varying concentrations of retinol (A) and RA (B). At the end of each treatment, CAT activity was measured as described in Section 2. The effect of antioxidant treatment with Trolox (100 μM) was evaluated with retinol at 7 and 14 μM (A) and RA at 100 nM and 1 μM (B). Values represent means ± SEM of four independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, compared to control; $^#p < 0.05$, compared to * and ** groups, as analyzed by ANOVA (Tukey's post hoc).

Next, we evaluated if the changes in CAT activity by retinol and RA were related to the modulation of CAT expression. We first evaluated the immunocontent of CAT at different time points during the 24 h period of incubation using a specific anti-CAT antibody. Retinol 7 μM increased CAT content only at 12 and 24 h (61% and 57%, respectively), and this effect was reversed by Trolox (Fig. 3A). No differences were observed with different concentrations of RA at any time points analyzed (Fig. 3B).

To establish if the increase in CAT immunocontent by retinol 7 μM observed at 12 and 24 h of incubation was resulting from increased mRNA transcription, we evaluated CAT mRNA expression by RT-PCR. No statistically significant differences in CAT mRNA expression by retinol were observed (Fig. 4). We also verified CAT mRNA expression in cells incubated with RA 100 nM and 1 μM, and both concentrations did not alter CAT expression. Finally, to evaluate whether retinol or RA directly modulate CAT activity, we performed a set of *in vitro* experiments using commercial CAT purified from bovine liver. Neither retinol (2–14 μM) nor RA (1 nM–1 μM) induced any significant changes in CAT activity (Fig. 5).

4. Discussion

CAT, along with glutathione peroxidase (GPx), exerts a major role in metabolizing hydrogen peroxide in cells. Peroxisomes are the main site of CAT expression, where, under basal conditions, this enzyme exerts a major role in the cleaning of H_2O_2 generated by the peroxisomal β-oxidation. CAT and GPx enzymatic activity may be acutely enhanced by toxic concentrations of H_2O_2, while increased enzyme expression often results from chronic elevations of H_2O_2 and other reactive species (Johnson, 2002). For this reason, both enzymes are extensively used as an index of unbalanced reactive species production and oxidative stress in physiological systems (Matés, 2000).

The redox properties of vitamin A and related compounds have been the subject of intense debate for years (Mayne et al., 1991). Initially, retinoids were claimed to exert important antioxidant functions in biological systems, and this belief stimulated the use of retinoids in antioxidants and nutritional supplements for prevention and treatment of diseases (Hix et al., 2004). However, the overly simplistic approach that supplementation of human diets with antioxidants is always beneficial has proven to be sometimes ineffective, such as the *N*-acetylcysteine-EUROSCAN (van Zandwijk et al., 2000), or even pathogenic, like the CARET trials in USA and ATBC trials in Finland (Omenn et al., 1994; The ATBC Cancer Prevention Study Group, 1994).

In Sertoli cells, retinol metabolism under physiological conditions is intense. Retinol is stored in cytoplasmic lipid droplets as retinyl esters, or is converted into RA through two enzymatic steps (Silva et al., 2002). It was generally accepted that this conversion into RA followed by RAR/RXR-mediated gene transcription in Sertoli cells was the main mechanism by which vitamin A exerted its hormonal function on spermatogenesis regulation (Sanborn et al., 1987). Nonetheless, the previous observations that both retinol and RA up-regulated antioxidant enzymatic activities (such as CAT) in Sertoli cells, and that these changes were accompanied by increased oxidative damage to DNA, lipids and proteins, suggested that, under certain conditions, retinol and RA exert pro-oxidant effects in cell systems (Dal-Pizzol et al., 2001b). These observations were compatible with other *in vitro* and *in vivo* studies where pro-oxidant activities of retinol and related compounds were reported (Murata and Kawanishi, 2000; Penniston and Tanumihardjo, 2006).

Here, we observed that specific concentrations of retinol and RA induced an increase in the production of intracellular reactive species, and this effect increased in the course of 24 h of incubation. These compounds also induced a later activation of CAT (at 12 and 24 h) at concentrations similar to their pro-oxidant concentra-

A — Effects of retinol on CAT immunocontent
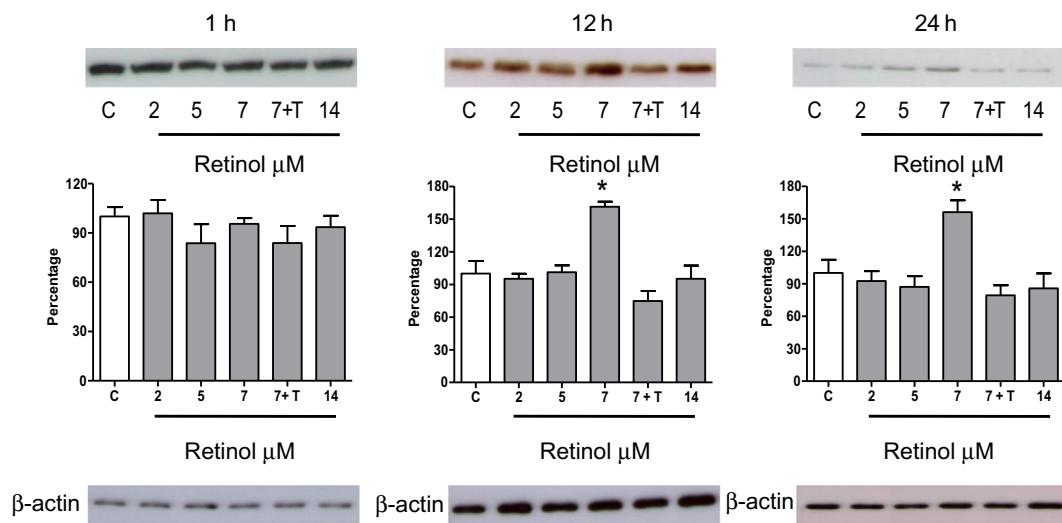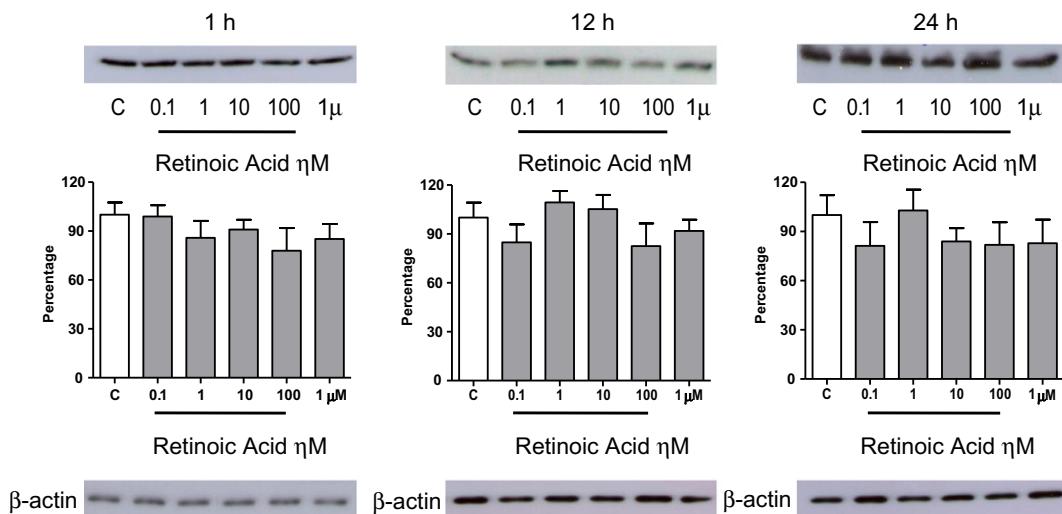
Fig. 3. Catalase immunocontent in cells treated with retinol and RA. Representative immunoblots with respective densitometric analyses showing CAT immunocontent in cells treated with varying concentrations of retinol (A) or RA (B) by 1, 12 and 24 h. Data were normalized in relation to β-actin content and are expressed as means ± SD for three individual experiments. $n = 3$; *statistically different from control ($p < 0.05$), ANOVA (Tukey's post hoc).

Effects of retinol or RA on CAT mRNA levels
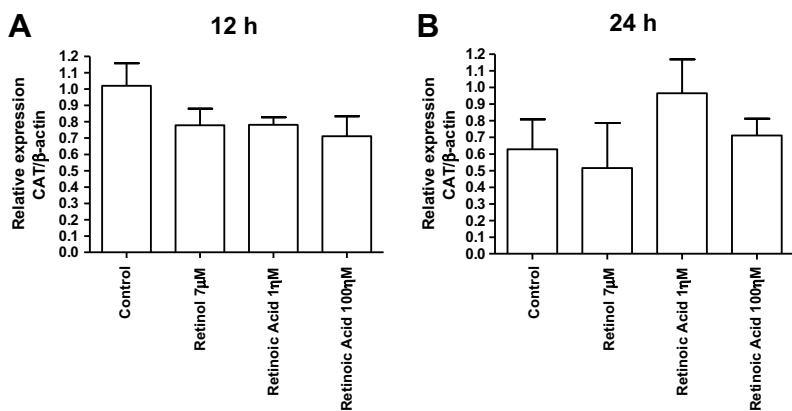
Fig. 4. Determination of CAT mRNA levels. RT-PCR analysis of Sertoli cells treated with retinol (7 μM) and RA (1 and 100 nM) for 12 (A) and 24 h (B). Data are expressed as means ± SEM $n = 6$; ($p < 0.05$), ANOVA (Tukey's post hoc).

55
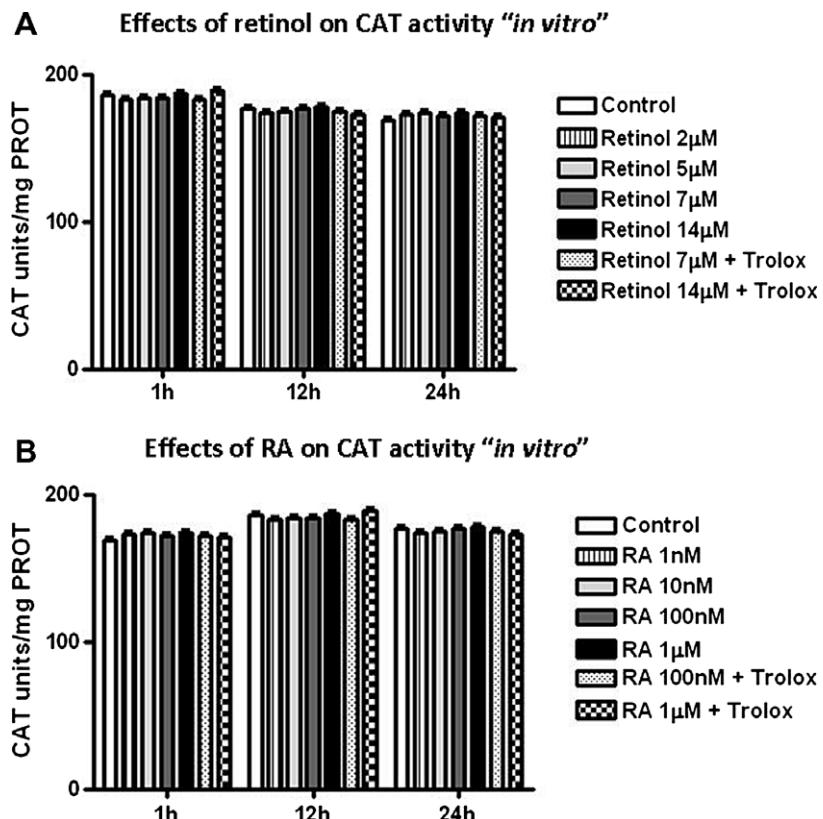
Fig. 5. Effect of retinol and RA on the activity of native CAT purified from bovine liver. Purified CAT from bovine liver (1 mg/mL) was incubated in 50 mM phosphate buffer (pH 7.0) at 25 °C in the dark for 1, 12, or 24 h with varying concentrations of retinol (A) or RA (B) in the presence of absence of Trolox® (100 μM), and enzyme activity was assayed. Data are expressed as means ± SEM $n = 6$; no differences between groups ($p < 0.05$) were detected by ANOVA (Tukey's post hoc).

tions, which suggested that the increased intracellular reactive species production by high levels of retinol and RA caused CAT activation. This resulted to be true for retinol, as the antioxidant Trolox inhibited both intracellular reactive species production and CAT activation, suggesting that CAT activity was enhanced in response to increased substrate availability caused by retinol-induced free radicals generation. However, although Trolox inhibited RA-induced formation of reactive species, it had not effect on CAT activation, indicating that RA-induced reactive species and CAT up-regulation were caused by distinct mechanisms. Moreover, although retinol at 14 μM increased reactive species production at a similar level of retinol 7 μM, increases in CAT activity and immunocontent were not observed with retinol 14 μM. This is probably because retinol at 14 μM causes a decrease Sertoli cell viability, leading to cell detachment and loss of protein content, as we observed in previous works (Gelain et al., in press; Dal-Pizzol et al., 2001b; Klamt et al., 2003). In a previous work, we reported that RA at 1 nM increased CAT activity by approximately 27% in Sertoli cells, and this was significantly different from control according to the student t test analysis (Conte da Frota et al., 2006). Here, we had a similar variation in CAT activity by RA at 1 nM, but a much higher increase was induced by higher concentrations of RA (100 nM–1 μM), and all groups were compared to each other using one-way ANOVA with Tukey's post hoc test, therefore a significant difference between the control and the RA 1 nM groups was not detected.

As stated above, antioxidant enzymes may be regulated by short-term activation (acute increase in enzyme activity due to allosteric or substrate activation) or long-term activation (increased expression due to a chronic condition of unbalanced reactive species production). As acute activation of CAT was not

observed at 1 h, we investigated how retinol and RA influenced CAT expression, and we observed that only cells treated with retinol had increased CAT immunocontent after 12 and 24 h of treatment, with unaltered CAT mRNA expression. This data suggested that retinol and RA effects on CAT were mediated by a non-classical mechanism of action, i.e., not involving RAR/RXR-mediated gene transcription, as these nuclear receptors are only activated by different isomeric forms of RA (which had no effect on CAT expression) and not by retinol itself. Moreover, it indicated that, in addition to a reactive species-independent mechanism, RA-induced CAT up-regulation occurred by a mechanism not related to protein expression regulation as well. The possibility that retinoylated proteins (proteins with RA molecules linked to their side-chains) act in ways not involving gene regulation is also an attractive hypothesis (Takahashi and Breitman, 1989; Breitman and Takahashi, 1996; Myhre et al., 1996; Renstrom and DeLuca, 1996), as well as other forms of direct allosteric interactions with CAT, such as the case of compounds like aminotriazole (Chang and Schroeder, 1972). However, we observed that both retinol and RA in vitro incubation by 1, 12, or 24 h did not affect CAT activity.

Our data showing the reversal of CAT increased immunocontent by Trolox in retinol-treated cells reinforced the suggestion that retinol-induced intracellular reactive species formation is the driving mechanism by which retinol up-regulates CAT. There are several redox-sensitive pathways that result in the transcription of genes involved in antioxidant response, such as NF-kB and Nrf2-regulated pathways. However, as we observed, retinol did not affect CAT mRNA expression, suggesting that non-genomic mechanisms were involved in this regulation. Decreased CAT turnover caused by oxidative stress is a possible mechanism. Intracellular reactive

species production induced by retinol may prevent CAT from pro-teasome-mediated degradation by promoting a change of the enzyme to a conformation called compound II (Kirkman et al., 1987; Kirkman and Gaetani, 2006), during exposure to its own substrate H_2O_2. These post-translational modifications as well as other possible non-genomic mechanisms of CAT modification by retinol-induced reactive species production may be further investigated in future studies.

In summary, our results show that retinol and RA activate CAT by different mechanisms, both not involving modulations of CAT mRNA levels; however, retinol increase CAT activity and immuno-content by a redox-dependent mechanism, while RA modulates CAT activity by a mechanism not dependent on reactive species production and not related to protein and mRNA expression. These data suggest the importance of keeping retinoid status within the physiological range, and reinforce the necessity of a better understanding of the redox properties and non-classical actions of vitamin A and other retinoids on biological systems.

Acknowledgements

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